Laboratory Technician's Manual

Medical Department
Enlisted Technicians School
Brooke General Hospital

Ft. Sam Houston, Texas
Third Edition, 31 May 1944.

1290—SAASFD—6-5-44—250

U. S. Army. Brooke general hospital,
" Fort Sam Houston, Tex.

L A B O R A T O R Y   B O O K

LABORATORY TECHNICIANS MANUAL

THIRD EDITION

TABLE OF CONTENTS

PART I

COMPILED BY LABORATORY SECTION
PRINTED AND BOUND BY PUBLICATIONS SECTION
MEDICAL DEPARTMENT ENLISTED TECHNICIANS SCHOOL
BROOKE GENERAL HOSPITAL, FORT SAM HOUSTON, TEXAS
31 May 1944

MICROSCOPE

The microscope is an instrument which magnifies minute objects for visual inspection, and is to be considered an instrument of precision.

Binocular microscope is a microscope with two eye pieces permitting the use of both eyes.

A compound microscope is one that contains two or more lenses.

Simple microscope is one that consists of a single lense or of several lenses that act as one.

The constituent parts of a laboratory G.I. compound microscope are:

1. Eye piece: may be of any magnification, the usual ones are:
    a. 6X.
    b. 10X.
2. Body tube: the body of the microscope.
3. Draw tube: an extending tube at the upper end of the body tube.
4. Pinion Heads.
    a. Coarse adjustment.
    b. Fine adjustment is made by the micrompter head.
5. The Rack: is a type of gear attached to the tube and is the means by which the pinion can make the adjustments.
6. The Revolving Nose Piece: is at the lower end of the body tube and holds the various objectives.
7. The objective is a group of lenses contained in the small piece at the lower end of the body tube.
    a. Lower power.
    b. High power (dry).
    c. Oil immersion.
8. The stage is that part of the microscope on which is placed the object that is to be viewed.
9. Mechanical stage is the mechanism attached to the stage which enambles by the use of pinions the movement of the object which is being viewed with accuracy and ease.
10. The condenser is the mechanism under the stage, the lenses of which focus the light on the object on the stage. Also contains an iris mechanism by which the quantity of light can be accurately controlled.
11. The mirror is below the stage at some distance and contains both a concave and a flat surface.
12. The entire mechanism is held together by the handle, pillar and base of the microscope.

MICROSCOPE

The microscope is an instrument which magnifies objects beyond the visual [illegible], and is to be considered an instrument of precision.

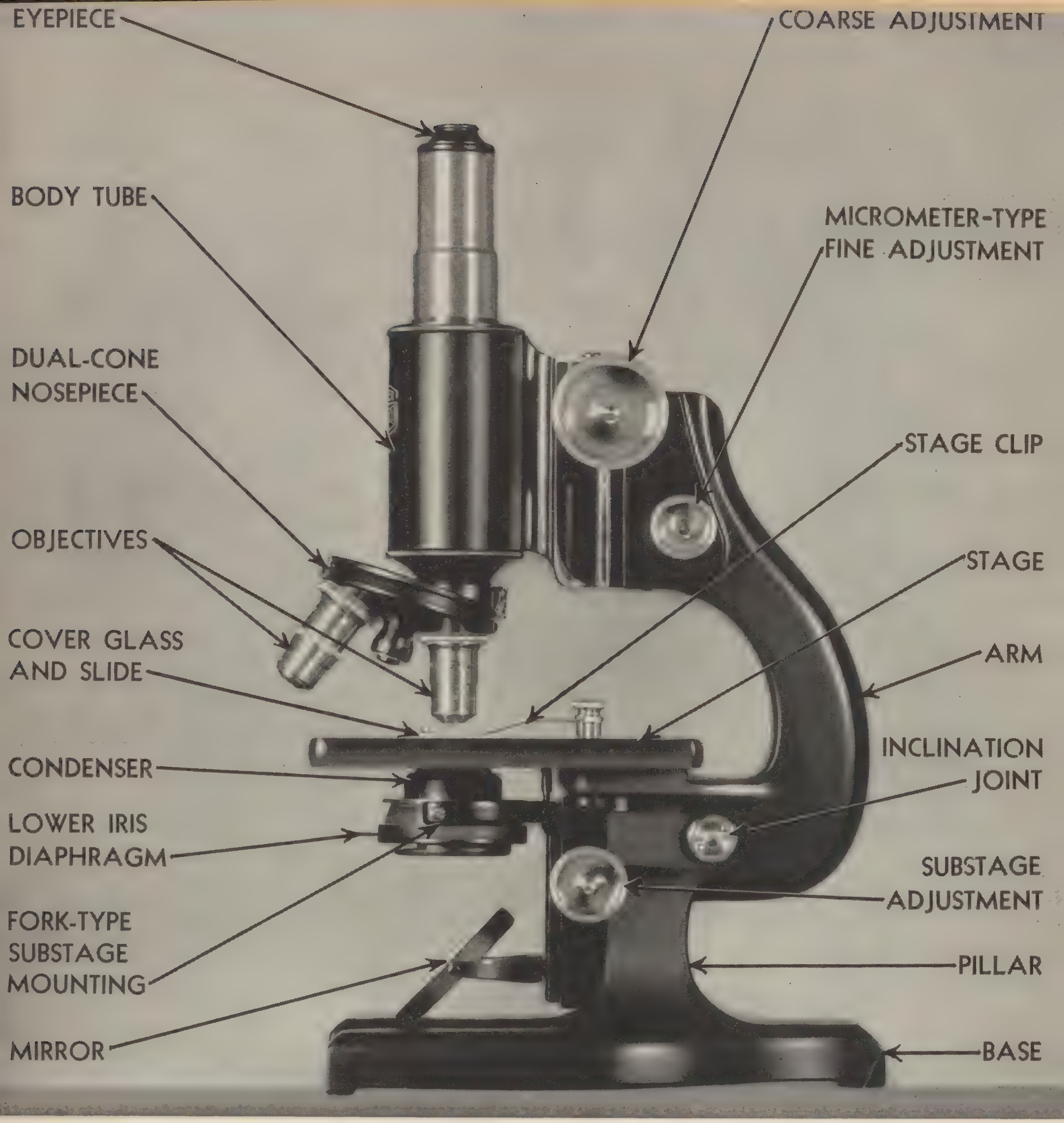
Binocular microscope is a microscope with two eye pieces permitting the use of both eyes.

A compound microscope is one that contains two or more lenses.

Simple microscope is one that consists of a single lens or of several lenses that act as one.

The constructional parts of a laboratory [illegible] compound microscope are:

1. [illegible] may be of any magnification, the [illegible] are:
 a. [illegible]
 b. [illegible]
2. Body tube the body of the microscope.
3. Draw tube an extension tube at the upper end of the body tube.
4. Pinion Heads.
 a. Coarse adjustment.
 b. Fine adjustment is made by [illegible].
5. The [illegible] is a type of lens attached to the draw tube and is the means by which the [illegible]
6. The nosepiece [illegible] is at the lower end of the body tube and holds the various objectives.
7. The objective is a group of lenses contained in the small [illegible] at the lower end of the body tube.
 a. Low power [illegible]
 b. High power [illegible]
 c. Oil immersion.
8. The stage is that part of the microscope on which is placed the object that is to be viewed.
9. [illegible] is the [illegible] which [illegible] the [illegible]
10. The diaphragm is [illegible] under the stage. The [illegible] of [illegible] light on the object on the stage. [illegible] in which the quantity of light can be [illegible] controlled.
11. The mirror is below the stage to [illegible] light and has a concave and a flat surface.
12. The [illegible] is held together by the [illegible], pillar and base of the microscope.



Mechanical Features of the Microscope

METHOD OF CALIBRATING MICROMETER DISCS FOR EYEPIECES

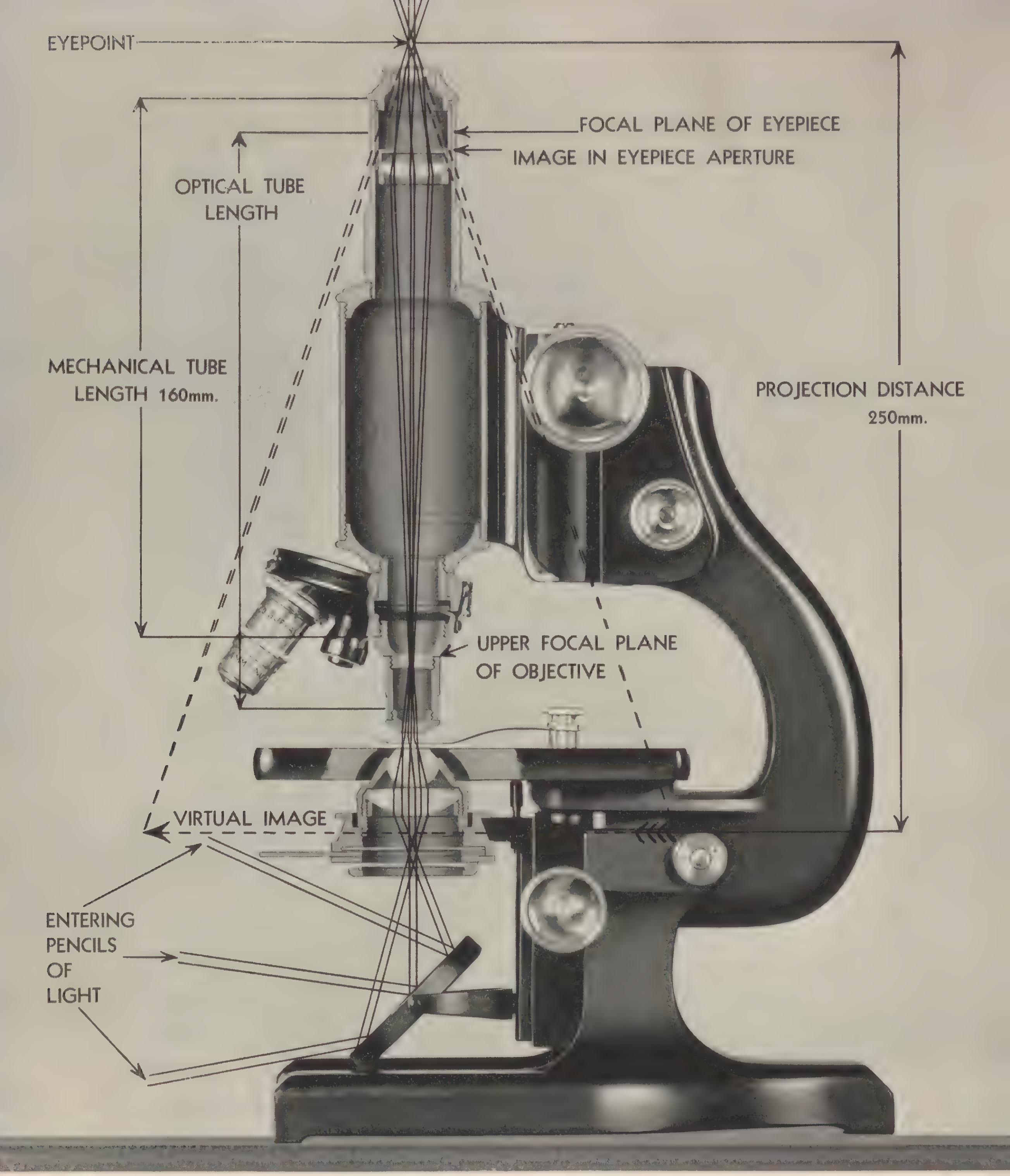
First, place the micrometer disc in the proper position in the eyepiece. The engraved scale should be downward or in the plane of the diaphragm. Place a stage micrometer (Cat. No. 400) beneath the objective. Count the number of divisions of the image of the stage micrometer that lie between either adjacent lines of the eyepiece micrometer or a known number of graduations of the eyepiece micrometer. It is preferable to use as many graduations of the eyepiece micrometer as is convenient.

DIVIDE THE LENGTH USED OF THE STAGE MICROMETER BY THE LENGTH USED OF THE EYEPIECE MICROMETER.

It is important to recognize that the graduations of the stage micrometer usually are hundredths of a millimeter and the graduations of the eyepiece micrometer usually are tenths of a millimeter or twentieths of a millimeter. As an example, if an eyepiece micrometer that is graduated in tenths of a millimeter is used, and five divisions of the eyepiece micrometer are found to take in two and one-half divisions of the stage micrometer, then what is usually called the reduction factor of this eyepiece micrometer is determined by the fraction $\frac{.025,}{.5}$ which is .05.

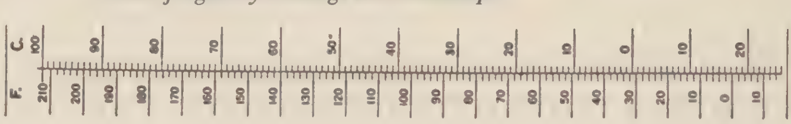
This reduction factor must be determined for each combination of objective and eyepiece that is used. Once determined it can be used continually with this combination, with the same length of draw tube.

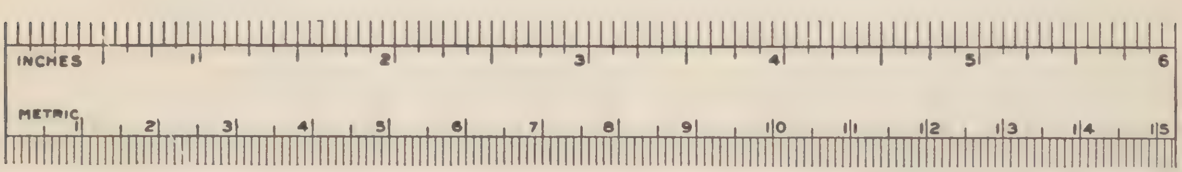
In using this factor, the number of graduations of the eyepiece micrometer, that measure the length of an unknown object on the stage, is determined. This number of graduations is multiplied by the reduction factor, giving the actual length in millimeters of the unknown object.



Path of light rays through the Microscope

Temperature Conversion Scale





M-81-IV 343

PRINTED IN U. S. A.

ANATOMY AND PHYSIOLOGY

I. Definitions.

1. Cell - a cell is the simplest unit from which all living things are built. Each cell has an outer membrane, cytoplasm and a nucleus.
2. Tissue - a tissue is a group of cells, similar in origin, structure and function, together with the substance between the cells.
3. Organ - an organ is a group of tissues which are united together in one unit for the performance of a special function or work.
4. A system - a system is a group of organs associated together to perform a special function (work).
5. Anatomy - anatomy is the study of the structure of the body and the relation of the different tissues and organs of the body to one another.
6. Physiology - physiology is the study of the workings of the various organs and systems of the body, both independently and together in life.

II. The Systems.

1. The Skeletal System is composed of bones and joints. The skeleton is the bony framework of the body, gives it stability and form, and protects the organs, while the joints permit of motion. Bone is composed of about one third animal matter, mostly gelatin, and two-thirds mineral matter, chiefly lime salts. The animal matter gives bone its toughness and elasticity, while the mineral matter gives it its hardness. Bones may be classified as long, short, flat and irregular.

   The long bones, of which the thigh bone, and shin bone are examples, form a system of levers which support the weight of the body and provide means of moving about. The short bones, as those of the wrist and ankle, are found where strength and limited motion are needed. The flat bones, as those of the skull, serve chiefly for protection. The irregular bones are illustrated by those of the pelvis.

2. The Muscular System is composed of muscles. The muscles provide the power to move the bones and thus make it possible for us to move about, pick up food, chew it and swallow it and so forth. Muscles have the power of contracting only, as a rubber band. They cannot push or shove, only pull. What then stretches them? For each muscle that pulls one way, there is another muscle which pulls in the opposite direction by system of levers and pulleys. For example, take the flexors and extensors of the forearm. The flexor muscles flex or bend the forearm at its joint, the elbow, while the extensors straighten or extend the forearm. There are, of course, other types of muscles which can only be mentioned and not explained, because of the shortness of the time. They are abductors, adductors and rotators. The aforementioned muscles all

come under the general heading of voluntary muscles or those moved at will. The involuntary muscles are those like the heart and muscles of the stomach and intestine, which work without our thinking about them.

3. The Nervous System is composed of the brain, spinal cord, nerves and ganglia. This is a very complicated system (far beyond the scope of this course) which enables man to think and to make the complicated machine, which our body is, work together as a coordinated whole. The brain, situated within the cranium, is the "seat" of all intellect and will, and the central station from which all the orders for motion are sent out and to which all the reports called sensations are forwarded. The spinal cord extends downward from the brain through the spinal canal and is largely a bundle of nerves or wires like a big cable. The nerves branch off from the spinal cord to all parts of the body. The ganglia are small masses of nervous tissue arranged in pairs along the spinal column and in groups about the heart and great viscera; they have to do with the involuntary musculature of the heart, lungs, blood vessels, gastro-intestinal tract, and the great viscera.

When a person wants to walk, for example, messages are sent from the brain down the spinal cord, out the nerves to the muscles of the legs and feet; also by different nerves to the muscles of the arms, so that they will swing with the legs; and by yet other nerves to the muscles of the back for balance.

The special senses are special modifications of the nervous system. They are - touch, located generally in the skin, but more especially in the finger tips; taste, smell, hearing and sight.

4. The Circulatory System is made up of the heart, blood vessels, lymph vessels, the blood and the lymph. This system brings food and water to the various organs, tissues and cells, as well as carries off their waste products. The driving power, of course, for all of this is the heart, the great pump. The human heart, as well as the heart of the higher animals, is a four-chambered muscular organ, which weighs about 3/4 of a pound. It may be divided into a right and a left side, each with two chambers. The right side receives the impure blood returning from all parts of the body, and forces it through the pulmonary circulation in the lungs, where the blood is purified. The left side of the heart receives the pure blood from the lungs and forces it out the great artery called the aorta, which breaks up into numerous smaller arteries, taking the blood to all parts of the body. The right auricle or atrium has a thin muscular wall and acts chiefly as a reservoir to receive the impure blood returning through the great veins. With the contraction of the auricle, the valve leading into the right ventricle is opened and the blood flows into the right ventricle. Very shortly afterwards the thicker walled right ventricle contracts. This closes the valve between the ventricle and

the auricle, at the same time opening the valve in the pulmonary artery, so that the blood is forced out the pulmonary artery to the lungs. The left auricle or atrium then receives the blood from the lungs through the pulmonary veins and when it contracts the blood passes through a valve into the left ventricle. The left ventricle, since it has to force the blood to all parts of the body, has a thicker muscular wall than the right ventricle. When the left ventricle contracts it closes the valve to the left auricle and opens the valve into the aorta and forces the blood out. Although the right and left sides of the heart have been discussed separately, it must be remembered that both auricles contract together and both ventricles about 0.16 of a second later.

The blood vessels may be classified as <u>arteries</u>, <u>capillaries</u> and <u>veins</u>. The arteries carry blood away from the heart; they have muscular and elastic walls. The capillaries are fine caliber, thin walled vessels which form a network for the exchange of food, water and gases, with the cells of the tissues and organs of the body. The veins carry the blood back to the heart. The veins differ from the arteries in that they have valves in their walls to prevent the back flow of blood and also have thinner muscular walls with less elastic tissue. The capillaries form a bridge between the small branches of the arteries and the smaller veins. Later, when you prick fingers for blood, it is from this capillary network that the blood will come.

The lymph vessels are entirely separate from the blood vessels and carry a thin white milky fluid called lymph. We cannot take time to discuss this here. The composition of the blood will be taken up in another lecture.

5. <u>The Respiratory System</u> is composed of the air passages, the nose, mouth, pharynx, larynx (voice box), trachea (wind pipe) and the lungs. The respiratory system is very closely associated with the muscular and circulatory systems. It is the muscular system, chiefly the diaphragm, which causes the air to be drawn into the lungs and it is the circulatory system which effects the exchange of carbon dioxide gas and water vapor for the oxygen in the fresh air.

The diaphragm is a dome-shaped muscle (top of the dome up) which lies between the thoracic cage (in which the lungs and heart lie) and the abdomen. The diaphragm sucks air into the lungs very much the same as the plunger in a pump sucks air into a pump. Since it is dome-shaped, when the diaphragm contracts, it flattens out and pulls down like the plunger. Since the lungs are very elastic they expand as the air rushes in. At the same time the lungs expand laterally as the inside of the thoracic cage is made larger by the expansion of the chest. The lungs are two in number, a right and a left. The right lung is divided into three lobes: upper, middle and lower lobes, while the left lung is composed of two lobes only: the left upper and left lower lobes. The lungs may be compared to a fine sponge. In the thin walls separating the numerous air spaces, the pulmonary

capillaries run so that there is only a very thin wall between the blood and the air. Since there is relatively much oxygen and very little carbon dioxide and moisture in the fresh air in the lungs, while there is the reverse, that is, more carbon dioxide and moisture and less oxygen in the impure blood, there is an equalization which takes place through the thin membrane separating the blood and air. The oxygen passes into the blood while carbon dioxide and moisture pass out into the air in the lung spaces. In this manner, the blood is purified.

6. The Digestive System is made up of the alimentary canal (30 feet), the mouth, pharynx, esophagus or gullet, stomach, small intestine and large intestine; and accessory organs which produce digestive juices as the liver, pancreas and salivary glands. Since the nutritive constituents of the blood are constantly being used up in the repair of tissue and the production of energy, either as heat or work, it is necessary that there should be a constant supply of new material. This is done by the digestive system which takes food and breaks it down into simpler products for absorption into the blood. Foods may be classified into five headings according to the alimentary principles which they contain:
   a. Proteins or nitrogenous substances.
   b. Fats.
   c. Carbohydrates or starches and sugars.
   d. Minearals including water and salts.
   e. Accessory food substances or vitamins.

   All of these alimentary principles are necessary for life.

   In the mouth, provision is made for the mastication or chewing of the food and its admixture, with saliva; beyond this is the apparatus for swallowing, the pharynx and esophagus, which convey the food to the stomach, where a partial reduction and solution of it takes place. In the small intestine the digestion and solution are completed with the aid of bile salts from the liver and pancreatic juice from the pancreas. The nutritive principles, composing the chyme (digested food) are taken up by the blood capillaries which lie in the intestinal membranes. The unabsorbable or undigested material together with a large amount of water, pass on into the large intestine where water is absorbed into the blood, thus leaving a more dry waste or formed feces which is expelled through the anus. The spleen has no direct part in digestion, but it does serve indirectly by acting as a reservoir for the storage, in the intervals of digestion, of the additional amount of blood needed during digestion. Other important functions of the spleen are the production of leucocytes, or white blood cells, and the destruction of erythrocytes or red blood cells.

7. The Excretory System is composed of the kidneys, ureters, bladder, urethra, and to a less extent, skin, lungs and intestine. In all life processes waste products and poisons are produced, which, if not eliminated, are finally

fatal even to the life which produced them. The yeast fungus growing in a sugar solution produces a poison, alcohol, which when it reaches a certain concentration, destroys the life of the yeast; so with the human, it produces poisons which must be thrown off if the body would live, and the apparatus by which these poisons are eliminated is known as the excretory system.

The kidneys, one on each side, are situated in the loins, at the back of the abdomen, on either side of the spinal column, and just below the last rib. They are about four inches long, and weigh about five ounces each. They consist of two portions, a cortex and a medullary portion. The cortex or outer portion consists of numerous groups of capillaries called glomeruli where water and salts are filtered from the blood into the tubules. Farther down these tubules, the secreting epithelium, with which the tubules are lined, takes from the blood urea and other waste products to complete the urine. The medulla or inner portion of the kidney consists largely of a number of urinary tubules which collect the urine from the various units of the cortex and empty the urine into a funnel-shaped sac, situated in a depression on the inner side of the kidney, called the pelvis. The pelvis has a constricted neck which is the starting point of the ureter. The ureters are two musculo-membranous tubes about the size of a goose quill, which extend from the pelvis of the kidney to the urinary bladder.

The urinary bladder is a muscular bag situated behind the pubis and directly in front of the rectum. When moderately full it holds one pint and under certain conditions may be extended to hold a quart or more. The urethra is eight or nine inches long in the male and extends from the neck of the bladder to the meatus which is the external opening; surrounding the urethra at the neck of the bladder is a pear-shaped gland called the prostate. This can be felt through the rectum which is directly behind it. In gonorrhea or "clap" it is this gland, the prostate, which retains the gonoccocci or the causative organisms.

The excretions of the skin will be taken up later. The excretion of the lungs, moisture and carbon dioxide has already been mentioned. The excretion of the intestine, namely, feces, is only mentioned in passing.

The average man passes about fifteen hundred cubic centimeters or three pints of urine a day. The composition of urine will be taken up later.

8. The Reproductive System is included here only for completeness. In the male it consists of paired organs; the testes, the epididymis, the vas deferens, the seminal vesicles, the ejaculatory ducts; unpaired parts are the prostate, urethra and penis.

In the female they consist of the paired organs, the ovaries and oviducts; and the unpaired, uterus, or womb, the cervix and vagina.

9. The Endocrine System or the glands of internal secretion are the islet cells of the pancreas, thyroid gland, parathyroid glands, suprarenal or adrenal glands, the hypophysis cerebri or pituitary, and certain tissues of the gonads or testes and

ovaries. This system also is included only for completeness and need not concern us here. These glands secrete certain chemical substances, called hormones, directly into the blood which have to do with the growth and development of the body and its control in relation to its environment.

10. The Integument is the skin or covering of the body with its glands, sebaceous and sweat glands, and its derivatives, hair, nails and teeth. The skin is a tough, elastic membrane which covers the entire body and is continuous at the various orifices, as the nose, mouth and anus, with the mucous membrane. Anatomically it consists of two layers, the epidermis or cuticle, and the derma or true skin. The cuticle is that part which is raised when a blister occurs and which pulls off after Scarlet Fever. It serves as the protection for the true skin.

The derma constitutes the greater part of the thickness of the skin, and contains the blood vessels, nerves, sebaceous (oil) and sweat glands.

The sebaceous glands secrete an oily substance which gives to the skin its softness and pliability; the orifices of the ducts of the sebaceous glands are particularly large about the face and nose, and when plugged with dirt from the familiar "black-heads".

The sweat glands are in vast numbers all over the body and their orifices constitute what are known as the pores. They secrete a variable amount of water, averaging about two pints a day, and the water contains organic matter and salts, and constitutes the perspiration or sweat.

ANATOMY AND PHYSIOLOGY OF THE URINARY SYSTEM

Now that we are about to delve into the field of urine analysis, it is fitting that we should first learn a little about the anatomy and physiology of the urinary system.

To sum it up briefly, the urinary system consists of the kidneys, the ureters, the bladder and the urethra whose function it is to eliminate from the body certain nitrogenous wastes of the body diluted in water and to help regulate the acid base balance of the blood.

The kidneys, two in number, are located at the back of the abdomen, one on either side of the spinal column just in front of the short or floating ribs. The kidneys are bean-shaped and together weigh 300 grams or about 2/3 of a pound. Each kidney is covered by a thin connective tissue capsule and is surrounded by fat whose connective tissue holds the kidneys in place. The side of the kidney, directed toward the spinal column, is indented and is called the hilum. It is through the hilum that the artery and vein of the kidney enter. They are also called the renal artery and vein since "renal" refers to the kidney. Each kidney consists of a cortex and a medulla. The cortex is the outer portion in which is located the smaller blood vessels, the glomeruli and the small tubules. The medulla is the central portion in which the pyramids with their collecting tubules, and the larger blood vessels lie. A cross section of a fresh kidney appears light reddish brown. In the outer band, which is the cortex, numerous small red pin points are seen. These are the glomeruli. The pyramids of the medulla, so called because they are shaped like an inverted pyramid with their bases toward the cortex and their apices toward the hilum, are a little darker brown and are striated, the striations being the collecting tubules which converge toward the apex of the pyramid. Of course, there is connective tissue which holds the blood vessels, glomeruli, small and large tubules together. (See Figure I.)

Surrounding the apex of each pyramid is a cup-shaped structure made of connective tissue lined with epithelium, called a minor calyx, which receives the urine from the collecting tubules of the pyramids. Several of these minor calyces are received into a similar but larger structure called a major calyx. There are three of these major calyces which are received into a funnel-shaped structure called the <u>renal pelvis</u>, which lies partly within the hilum of the kidney. The renal pelvis consists of three layers, an inner epithelial layer, a middle muscular layer and an outer connective tissue layer.

The small end of the "funnel" of the renal pelvis is continuous with the ureter, which is a small structure about the size of a goose quill which extends down along the side of the spinal column at the back of the abdomen to the brim of the pelvic cavity where it passes along the lateral wall of the pelvis, then dips downward, then toward the mid line and finally upward to enter the bladder on its under surface. The two ureters enter the bladder about 1-1/2 inches apart. The ureter also consists of three layers: an inner epithelial layer, a middle muscular layer and an outer connective tissue layer. The muscular layer is composed of an inner longitudinal and an outer circular layer of muscle.

The urinary bladder is a muscular bag lined with epithelium which is situated behind the pubis and in front of the rectum. In the female the uterus is between the two. The muscular coat of the urinary bladder is composed of an inner longitudinal, a middle circular and an outer longitudinal layer of involuntary muscle. The ureters pass through the muscular wall of the bladder in a diagonal course, so that when the bladder contracts the ureters are shut off to prevent the back flow of urine to the kidneys.

The opening of the urethra at the front of the bladder with the two openings of the ureters form a triangle called the trigone of the bladder.

The urethra, which conveys the urine to the exterior, consists of a connective tissue tube lined with epithelium. In the female the urethra is slightly over one inch long, while in the male it varies up to 10 or more inches. The male urethra is subdivided into three parts: the prostatic urethra, which is completely surrounded by the prostate gland; the membranous portion, about one centimeter long between the prostate and the voluntary muscles in the floor of the pelvis, which form the external sphincter; and the longest part, the cavernous or penile urethra situated in the penis.

Now to go back again, we shall discuss in more detail the structure of the kidney so as to get an idea of the physiology of urine secretion. This can best be understood by a careful study of Figure II.

The renal artery and vein enter the hilum (indented portion of the kidney) just above the renal pelvis, where they soon break up into numerous branches. Now we shall follow the course of the blood.

The renal artery gives off numerous branches called interlobar arteries, which pass up between the pyramids. At the top of the pyramid the interlobar artery gives off a branch which curves off like an arch and is therefore called the arcuate artery. The arcuate artery gives off two types of branches: one, the interlobular artery, which is involved in the secretion of urine only; and, the interstitial artery which nourishes the tissues of the kidney.

The interlobular arteries pass up in the cortex giving off branches called the afferent arteries which break up into capillaries in the glomerulus. Unlike arteries elsewhere, the glomerular capillaries are received into arteries called efferent arteries. Each efferent artery again breaks up into capillaries about the proximal and distal convoluted tubules. These capillaries are finally received into the stellate veins. The stellate veins empty into the interlobar veins and so on back into the renal vein.

Bowman's capsule is really the beginning of the tubular system and is really the blind end of a tube which has been invaginated by the glomerular capillaries. A study of Fig. II will reveal clearly the tubular system.

RENAL FUNCTION

In the past 20 or 30 years there has been a great deal of work done on renal function. The complete story of the way in which urine is produced is still unknown. So far, we know that water, salts, uric acid, urea, creatinine, chloride and glucose are filtered out through the glomerular capillaries into Boman's capsule. In the tubules there is a

selective reabsorption back into the blood of glucose, chloride and water called threshold substances, by the cells lining the tubules, while non-threshold substances, such as uric acid, urea and creatinine are not re-absorbed and are passed on out in the urine. At the same time, the cells of the tubules form Ammonia from urea which, as the basic radical (NH_4) replaces the fixed bases, sodium and potassium, in the salts, so that the sodium and potassium is saved for the blood. This enables the kidney to eliminate acid radicals, partly neutralized by the (NH_4) radical, and thus helps to maintain the acid-base balance of the blood.

Normally the capillary walls are not permeable to the large protein or albumin molecules, but in disease when the supply of oxygen is low, the permeability of the capillary walls is altered, so that albumin passes through and we have albuminuria or albumin in the urine.

In diabetes mellitus, when sugar is not burned properly in the blood, due to the lack of insulin, the amount of sugar in the blood becomes very high. This sugar concentration exceeds the renal threshold so that sugar is excreted in the urine.

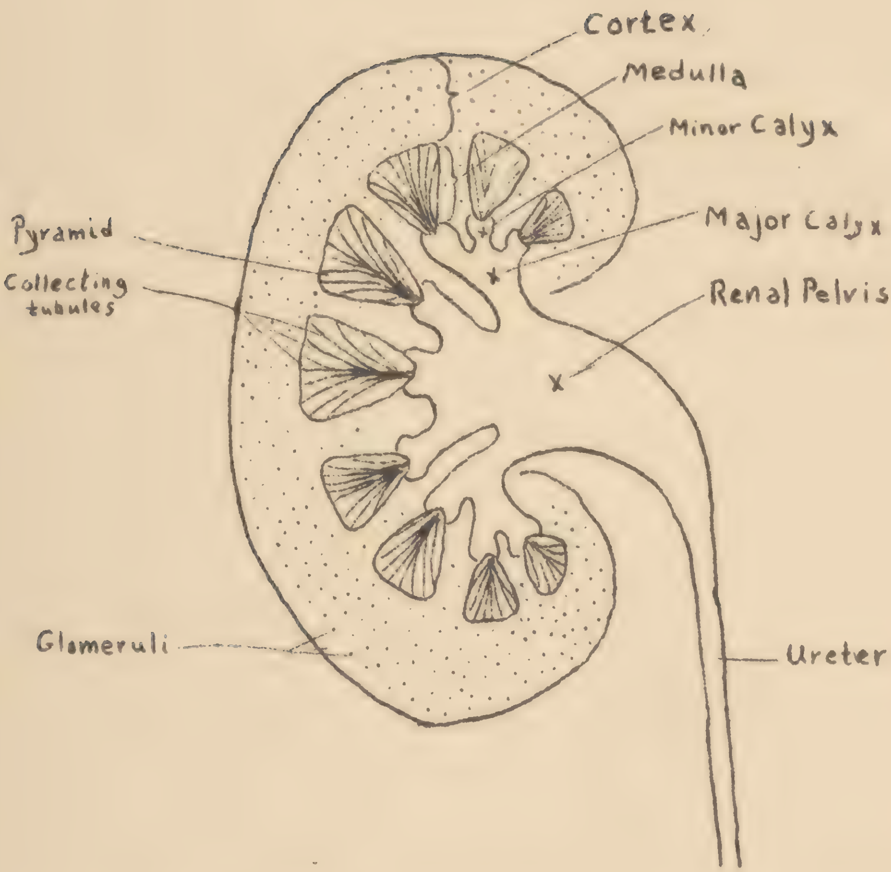
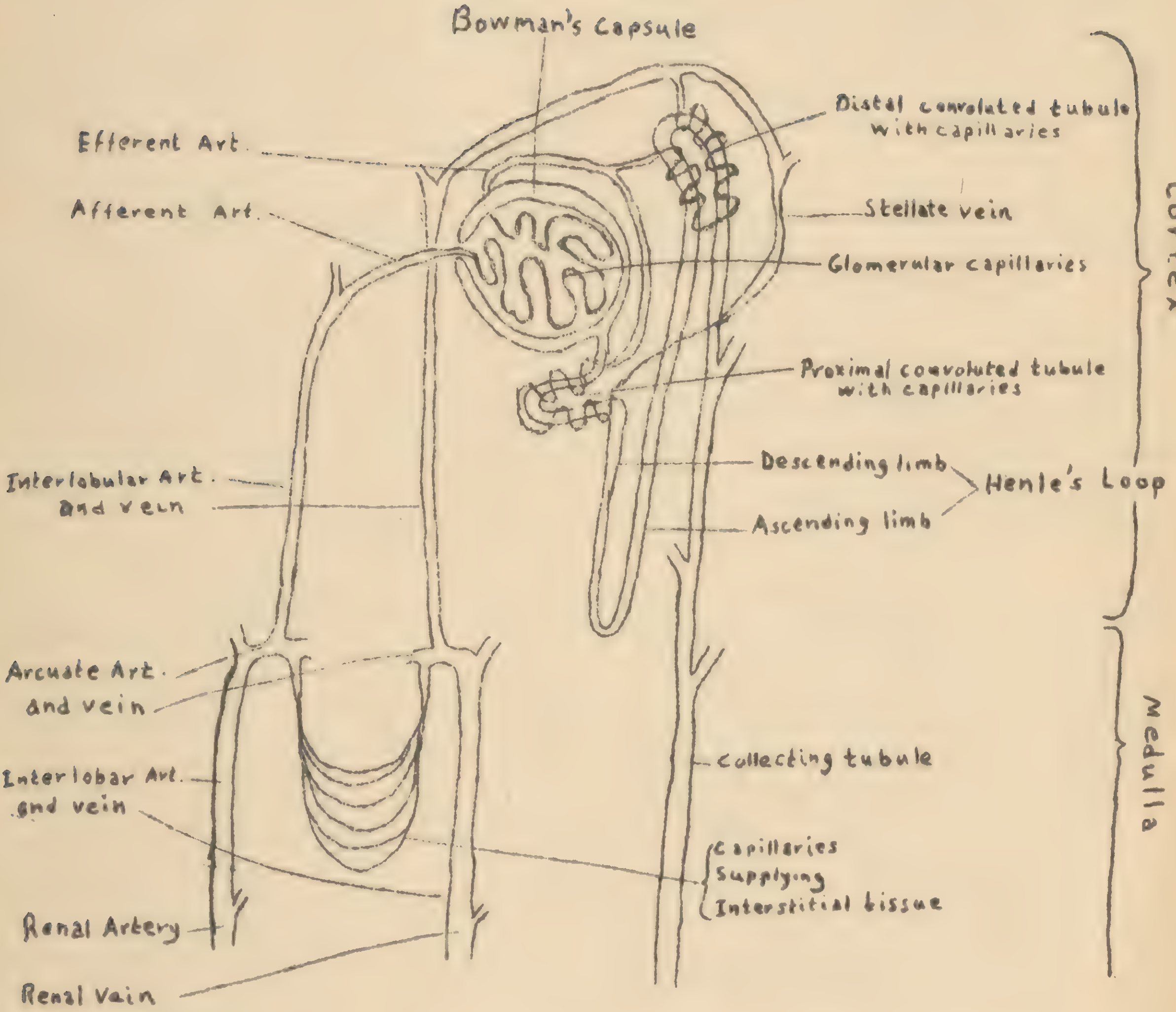


Fig. I – Schematic diagram of a longitudenal section of a kidney and ureter.



Glomerulus = Bowman's Capsule + Glomerular Capillaries

Fig II - Schematic diagram of the bloodsupply and tubuler system of a kidney.

ANATOMY AND PHYSIOLOGY OF THE DIGESTIVE SYSTEM

Now that we are about to take up the study of stool examinations, it is fitting that we take up in more detail the digestive system. By this time you should know that the digestive system consists of the alimentary canal and such accessory organs as those which produce digestive enzymes, or ferments, and assist in the handling of the food. The accessory organs include the teeth, tongue, salivary, gastric, and intestinal glands, liver and pancreas.

Alimentary Canal - This is a long tube, some 30 or more feet in length, which extends from the mouth to the anus. It is made up of the mouth, pharynx, esophagus, stomach, small and large intestine.

The general structural plan of the canal is: (1) a lining of mucous membrane, in which there are numerous glands; (2) a submucous layer of loose connective tissue, into which the glands may penetrate, and in which the blood vessels are located; (3) layers of involuntary muscular tissue (an inner circular, an outer longitudinal, and, in the stomach alone, an innermost oblique layer); and (4) and outer layer which is fibrous in nature above the diaphragm and serous below the diaphragm.

The Peritoneum - This may be defined as the serous membrane which lines the abdominal cavity and is invaginated (pushed in) by, or reflected over, the viscera. The different viscera in the abdomen lie within the peritoneum in the same manner as your fist would be within a partially inflated rubber balloon, if you were to push your fist into it. Layers of peritoneum which extend from the body wall to a viscus and suspend it, form the mesentery. The great omentum which covers the viscera like an apron is a long double fold of the peritoneum. Unlike the peritoneum elsewhere, however, the omentum contains a variable amount of fat tissue and lymphatics. The omentum affords protection for the viscera by its bulk, acting as a cushion, and through its power to move about it can help wall off infection such as from a ruptured appendix. For this reason the omentum is often called the "policeman" of the abdomen. See Fig. II and III.

The Mouth - The mouth is the beginning of the alimentary canal, it is composed of the vestibule and the oral cavity. The vestibule is that part between the lips and cheeks without, and the teeth within. The two communicate just behind the last molar tooth. The oral cavity is that part in front of the pharynx, and between the teeth, in which the tongue lies.

The teeth are composed of an outer hard substance called enamel which covers the exposed part, and a less hard substance called cementum, covering the root of the tooth, and the ground substance called dentine. In addition there is a central canal which contains the pulp tissue and the nerve. The full adult set of teeth is 32 in number or 28 without the wisdom teeth.

Tongue - The tongue is a mass of muscle covered with a rough pappilated mucous membrane. The tongue aids in chewing and swallowing the food and is important in talking. The sense organs of taste are located in the tongue.

Salivary Glands - These consist of three pairs of glands, namely: the parotid, the submaxillary and sublingual. The parotids are located, one in front of the lobe of each ear. It is inflammation of the parotid gland which we call mumps. The submaxillary glands are located in front of and below the angle of the jaw while the sublingual glands are under the tongue. The salivary glands secrete saliva which contains an enzyme, amylase. Amylase aids in the digestion of starches.

Pharynx - The pharynx is a muscular tube lined with mucous membrane which extends from the back of the nasal cavity to just below the thyroid cartilage where the esophagus begins. It communicates with the nose above; with the mouth in the middle, and with the larynx below. The pharynx is what is commonly called the throat.

Esophagus - The esophagus is a muscular tube lined with mucous membrane which extends from just below the thyroid cartilage to the upper end of the stomach. It passes through the lower part of the neck where it is between the trachea in front and the muscles overlying the vertebra behind. It passes through the thoracic cavity right in front of the vertebral column. In the lower part of the thoracic cavity it turns to the left slightly and passing in front of the aorta, it passes through a hole in the diaphragm, just to the left of the midline, to join the stomach on the abdominal side.

The upper 1/3 of the esophagus has a little voluntary muscle which is continuous with that of the pharynx. The musculature of the lower 2/3 consists entirely of involuntary muscle - an inner circular and an outer longitudenal layer. The mucous membrane of the esophagus contains mucus glands only, which lubricate the tube but have no direct connection with digestion.

The Act of Swallowing - In swallowing the bolus of food is flipped into the pharynx by the tongue, the nasopharynx being closed off by the soft palate, the muscles of the pharynx contract to pass the food on into the esophagus, where its involuntary muscles pass it on to the stomach. At the opening of the larynx there is a structure called the epiglottis, which in the act of swallowing, projects out over the opening of the larynx to prevent food from going down the trachea or wind pipe.

The Stomach - The stomach is a large muscular bag lined with mucous membrane, which is interposed between the esophagus and the small intestine. The upper end of the stomach, called the fundus, lies against the concavity of the diaphragm. See Figure I. Most of the stomach lies in the upper left quadrant of the abdomen. Its lesser curvature is in close relation to the liver. The lower end or phylorus is about in the midline and it joins the small intestine just to the right of the midline at the pyloric valve.

The mucous membrane or mucosa of the stomach is thrown up into numerous folds called rugae, which give more surface. The gastric glands lie in the mucosa; they are composed of two main types of cells: The parietal and chief cells. The parietal cells produce hydrochloric acid while the chief cells produce protease (pepsin). The muscular coat of the stomach consists of three layers: an inner oblique layer; a middle circular layer and an outer longitudinal layer. The middle circular layer becomes thicker at the pylorus to form the pyloric sphincter.

Small Intestine - The small intestine has the greatest length of the entire alimentary canal and is approximately 23 feet long. It has numerous coils. The small intestine is subdivided into three parts: the duodenum, the jejunum, and the ileum, from the top down. The small intestine also has a mucous membrane and a muscular coat composed of an inner circular and outer longitudinal layer of involuntary muscle. In the mucosa, chiefly of the duodenum, are the glands which produce the digestive enzymes or ferments, which complete the process of digestion. The mucosa is thrown up into numerous folds called plicae and these are further divided into projections called villi, all of which tend to increase the digestive and absorptive surface.

The lower end of the small intestine or the ileum, enters the large intestine in the right lower quadrant of the abdomen. This is called the ileo-caecal junction.

The Large Intestine - Starts in the right lower quadrant of the abdomen extends upwards along the right side of the abdomen to the liver, where it turns to the left, goes across to the left side, passing just below the stomach to the spleen where it turns downwards, passing along the left side of the abdomen to the pelvis where it takes an "S" curve toward the midline; then passes downward to the anus.

The large intestine or Colon is subdivided into several parts. The first part is a blind pouch, about 3 or 4 inches long, called the caecum to which the appendix is attached. The ileum enters the caecum at its upper end at the ileo-caecal valve. That part from the ileo-caecal valve to the liver is called the ascending colon. The bend at the liver is called the hepatic flexure, That part which extends across the abdomen to the spleen is called the transverse colon. The bend at the spleen is called the splenic flexure. That part from the spleen down to the pelvis is called the descending colon. The "S" shaped part in the pelvis is the sigmoid colon. The straight portion extending from the sigmoid to the anus is the rectum. The anus is the external opening only.

The mucosa of the colon appears smooth to the naked eye instead of being rough like the small intestine. It has, however, microscopically numerous muccous glands. The muscular coat has the usual inner circular and outer longitudinal muscular layers except that the longitudenal layer is concentrated into three narrow bands, visible to the naked eye, which are called tenia coli. At the lower end of the rectum the circular layers become thickened to form the internal (involuntary) sphincter. At the anus there is a sphincter of voluntary muscle.

Accessory Organs - The salivary glands have already been mentioned. The accessory organs situated in the abdomen are the liver and pancreas.

Liver - The liver is the largest gland in the body. It weighs normally 1400 to 1500 grams or about 6.5 lbs. The liver is closely applied to the under surface of the diaphragm. It consists of two main lobes, a right and a left lobe which are demarcated on the surface by the falciform ligament of the liver. The right lobe is the largest. The left lobe extends a short distance to the left of the midline.

Biliary System - The biliary system consists of the bile ducts both inside the liver and outside of it, and the gall bladder. The right and left hepatic ducts, one from each lobe of the liver join to form the common hepatic duct. The gall bladder, you might say, is a large outpouching of the common hepatic duct. The gall bladder is connected to the common hepatic duct by a narrow duct called the cystic duct. From the junction of the cystic duct and the common hepatic duct down, it is known as the common bile duct. The common bile duct and the pancreatic duct join to enter the duodenum together about 4 inches from the pyloric sphincter of the stomach. The gall bladder is a thin muscular bag lined with mucous membrane, where the bile, which is formed in the liver, is concentrated and stored until needed for digestion. In addition a thin mucus is added to the bile by the mucous glands of the gall bladder. See Fig. I.

Pancreas - The pancreas is a long narrow organ which normally weighs 90-150 grams or about one quarter of a pound. The pancreas lies at the back of the abdomen against the vertebral column in the upper part of the abdomen behind, and parallel with, the transverse colon. The head of the pancreas lies in the first bend of the duodenum and the tail lies against the spleen. The pancreas has two separate types of glandular tissue. One type secretes digestive juices into the pancreatic duct and the other type the islets of Langerhans, secrete insulin directly into the blood stream. Insulin is the substance which has to do with carbohydrate metabolism and it is the lack of it that causes Diabetes Mellitus. Thus, the pancreas is a gland of both internal and external secretion.

PHYSIOLOGY OF DIGESTION

Digestion is the process of getting foods into soluble form, so as to fit them for absorption into the blood, so that they may be carried to all tissues. This alteration is brought about by enzymes.

An enzyme is an organic catalyst, produced by a living organism. An enzyme increases the speed of a reaction without adding in any way to the energy changes involved in the reaction or taking part in the formation of the end products.

Enzyme action is specific. That is, a certain enzyme will act on one type of food stuff alone and no other. Enzymes also have an optimum temperature and optimum reaction. The optimum temperature of course is body temperature. The optimum reaction is acid for the gastric enzymes and alkaline for the intestinal and pancreatic enzymes.

The types of food may be classed as inorganic and organic. The inorganic foods are water and salts, which need no digestion. The organic foods are: carbohydrates (sugars and starches), fats, and proteins. In addition there are accessory foods (also organic) which are known as vitamins.

Digestion begins in the mouth by the process of chewing the food and the salivary enzyme amylase which begins the digestion of starch. After the voluntary act of swallowing, which has already been discussed, the bolus of food is passed down the esophagus by the involuntary muscles to the stomach by peristaltic waves. In the stomach gastric enzymes, protease and lipase, with the aid of hydrochloric acid, begin the breakdown of proteins and fats respectively. This is done as the food is churned about and mixed with the gastric juice.

The presence of food in the stomach sets up reflexes which cause bile to be released from the gall bladder and pancreatic juice from the pancreas into the duodenum. When the food in the stomach is well mixed with gastric juice the pyloric valve opens and the food passes into the duodenum.

In the duodenum and small intestine the food is mixed with the bile, intestinal and pancreatic juice and digestion is completed. The bile emulsifies the fats so that they can be broken down into fatty acids and glycerol by pancreatic lipase. The proteins are further broken down into amino acids by pancreatic and intestinal proteases. The digestion of starches and complex sugars into glucose is completed by various enzymes from both the pancreas and small intestine.

Now the fatty acids, amino acids and glucose are ready for absorption. This takes place through the small capillaries which lie in the villi of the small intestine. The majority of the fatty acids are taken up by small lymphatics, called lacteals, also situated in the villi.

The digested, as well as the undigested food and water, is passed along the small intestine by peristaltic waves. By the time the contents of the small intestine reach the ileo-caecal valve, digestion and absorption of the food products is complete.

In the large intestine the undigested and unabsorbable food is also passed along by peristalsis, but at a much slower rate; taking from 24 to 36 hours to pass through. In the large intestine the excess water is absorbed and more or less dry formed stools are produced. Anything which increases the rate of passage through the colon will give a watery stool or diarrhea.

There are numerous bacteria which normally live in the intestinal tract chiefly in the colon. These are chiefly the Bacillus Coli group and a few gas forming bacilli. As long as these bacteria remain within the intestinal tract, they do no harm, but, once free in the peritoneal cavity, as from a ruptured appendix or gun shot wound of the abdomen, they set up a very serious inflammation called peritonitis, which causes death more often than not.

Defecation. The dried feces, having accumulated in the rectum, sets up a defecation reflex. The internal sphincter is automatically released. At the convenience of the individual, the external, voluntary sphincter is released, the internal abdominal pressure is increased by tightening the voluntary abdominal muscles and the contraction of the diaphragm. At the same time, the levator ani, a voluntary muscle, which is attached to the anal or voluntary sphincter, is contracted, thus lifting the lower end of the rectum up over the formed stool so that it is expelled.

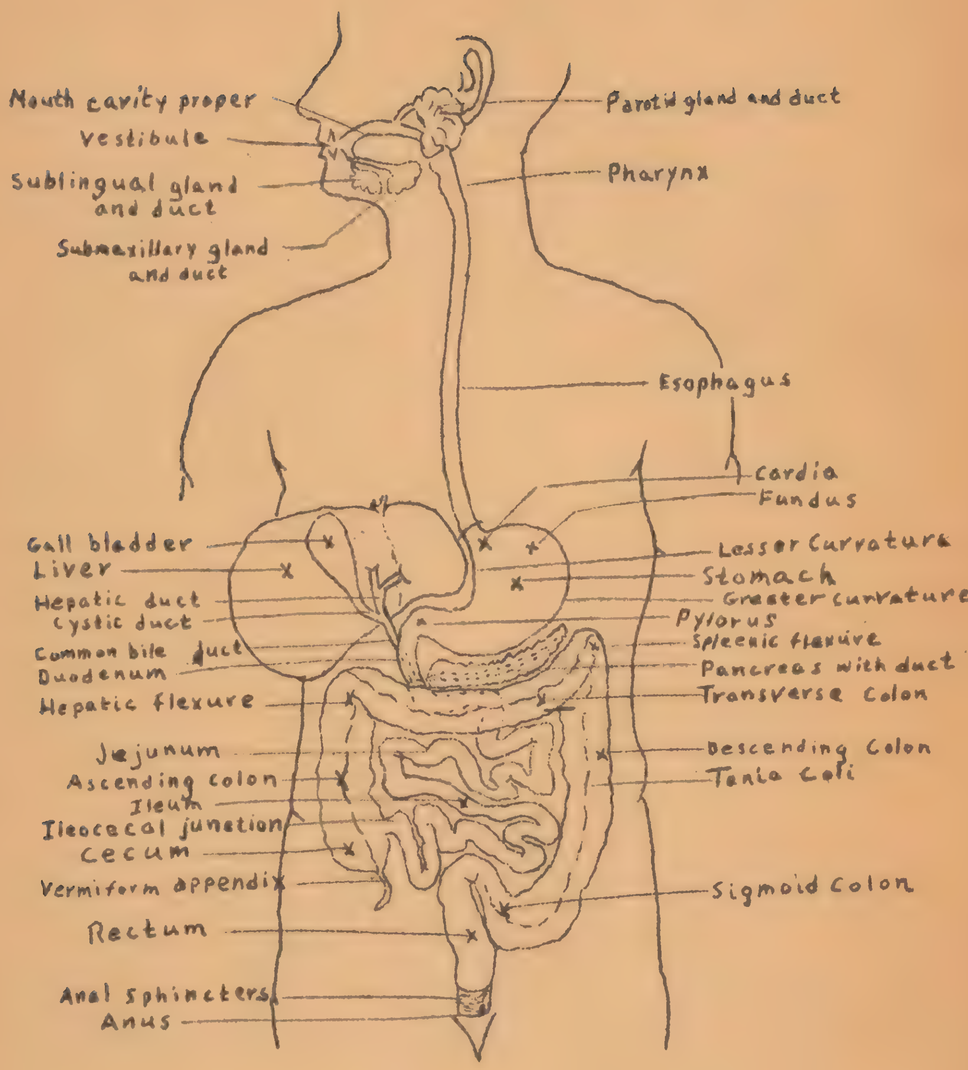


Figure I. A diagram of the digestive system, showing the alimentary canal and accessory organs. The liver and gall bladder have been turned up, toward the head and to the right.

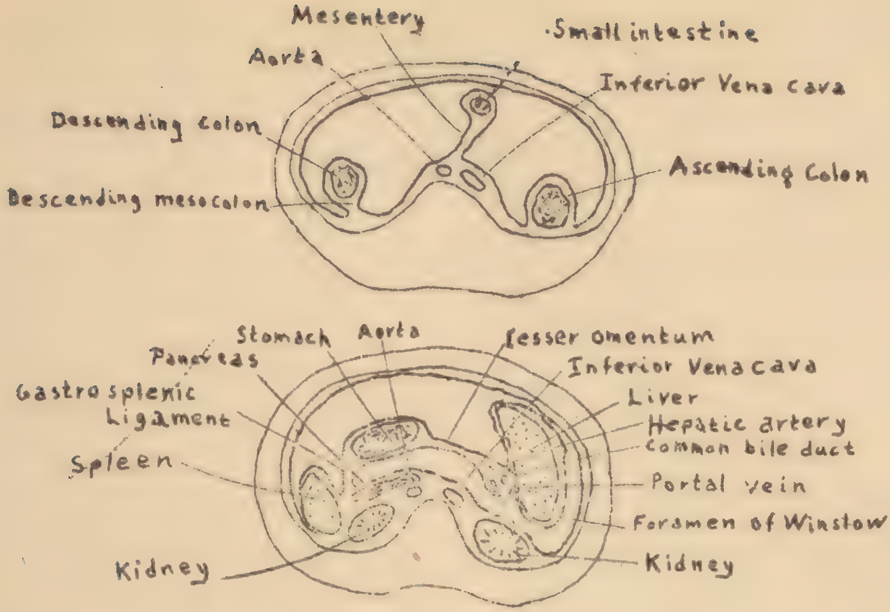


Figure II. Reflections of the peritoneum as seen in transverse section at two different levels.

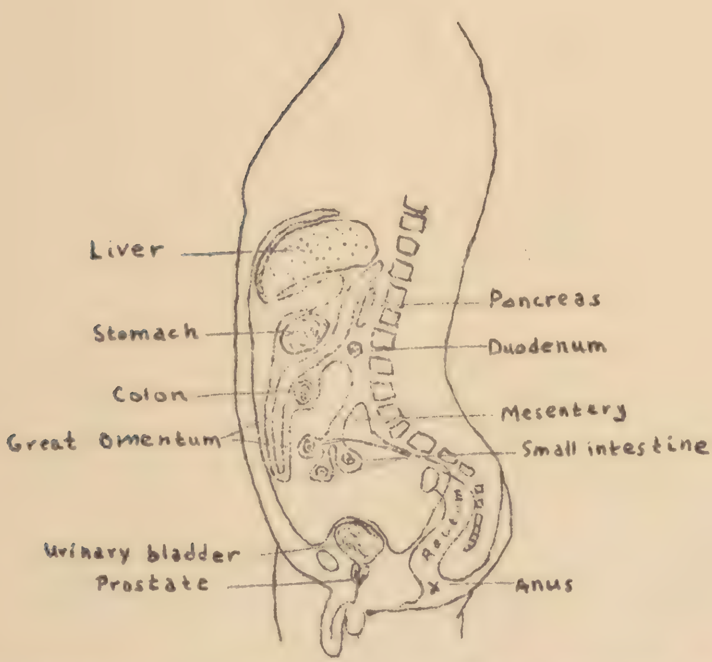


Figure III. Reflections of the peritoneum as seen in sagital section.

BLOOD

The functions of the blood and blood-vascular system are to receive from the lungs and alimentary tract and to carry to all parts of the body the materials necessary for its nutrition and proper temperature and moisture, and to carry away to the excretory organs the waste matters which, if retained, would prove poisonous. It has also important functions in the protection of the body from the invasion of the bacteria of disease.

The total quantity of blood is usually estimated at one-twelfth of the weight of the body, or an average of about a gallon and a half or six quarts.

The blood is red in color; bright red in the arteries; dark red in the veins. It is composed of cells or corpuscles floating in a liquid called the plasma.

The cells are of three sorts, the red cells or erythrocytes, the white cells or leukocytes, and the blood-plates or platelets. The red cells are much the more numerous, there being about five hundred times as many as there are white. The red cells are very small, about one three-thousandth (1/3000) of an inch in diameter, and, though red in mass, the individual cells are seen under the microscope to be light yellow in color. They are round, flattened discs, like a copper cent, except that they are concave on each side. They are composed largely of hemoglobin, a substance containing iron which has a great oxygen-carrying power.

Soon you will learn to count the red cells or erythrocytes. There are so many in the human body that it is beyond our comprehension; therefore, we count only a very small fraction of them and multiply by a factor which gives us an index of the number of red cells. The average number for women is four and a half million (4,500,000) per cubic millimeter and for men it is five million (5,000,000) per cubic millimeter. In order to impress upon you the total number of red cells in the body, assuming six quarts or approximately six liters for the total volume of blood, there are about thirty trillion (30,000,000,000,000) red cells in the body. If a person were to count one red cell per second, ten hours a day every day, it would take two million seven hundred and forty thousand (2,740,000) years to do it.

To get back to business, the leukocytes or white cells are not flat like a cent, but spherical like a ball, a little larger than red cells, composed of protoplasm, and capable of changing their own form and of making their way through the unbroken walls of the blood vessels. The leukocytes may be divided into two main groups: these are the non-granular leukocytes, or agranulocytes, which have no specific granules in their cytoplasm, and the granular leukocytes or granulocytes, which contain specific granules in their cytoplasm. Each group will be considered.

The non-granular leukocytes are divided into lymphocytes and monocytes. The lymphocytes are a little larger than the red cells; the distinguishing feature is a large round nucleus surrounded by a thin layer of blue cytoplasm in the stained smear. These constitute twenty to fifty percent of the total number of leukocytes.

The typical monocytes are a little larger than the lymphocytes. The nucleus is relatively large and usually indented; the cytoplasm is far more abundant than it is in the lymphocytes, has a pale blue, ground glass appearance in the stained smear and may have a few small red granules. These constitute three to eight per cent of the leukocytes.

The granular leukocytes always contain granules in their cytoplasm. The granules differ in various classes of granulocytes. The nucleus of the granulocytes is polymorphus (having many shapes) and is subdivided into lobes. The classes of granulocytes are: (1) acidophil or eosinophil; (2) basophil and (3) neutrophil leukocytes.

The eosinophils are slightly larger than lymphocytes. The nucleus usually has two oval lobes. The cytoplasm contains coarse, spherical granules which stain red. These cells constitute 1 to 4% of the leukocytes.

The basophils are the same size as the eosinophils, have a faint shadowy nucleus and are characterized by large, round, dark blue granules in the cytoplasm. These constitute 0.25% to 1% of the leukocyte count.

The neutrophilic leukocytes are about the same size as the eosinophils. The nucleus consists of two to five lobes in the adult cell and may be half moon band in the young cells. The cytoplasm contains fine granules which stain with neutral dyes and have a pale lavender color in stained smears. The neutrophils form fifty to seventy-five per cent of the leukocyte count.

The blood-plates or platelots are small, much smaller than a red cell, non-nucleated, circular discs. They tend to adhere to one another and to all surfaces with which they come in contact except the undamaged walls of blood vessels. The blood platelets play an important part in the coagulation of blood. They release a substance, which, together with tissue juice initiates the process. In stained smears the platelets appear as pale yellow or pink discs with fine blue granules. The average number is 800,000 per cu. mm. with a range of 500,000 to 1,500,000 per cu. mm.

Origin - in the embryo, the erythrocytes are formed in the blood islands in the liver and in the bone marrow. In the adult they are formed only in the red bone marrow. The granular leukocytes and platelets also originate from the red bone marrow, while the lymphocytes and monocytes originate from the lymphoid tissue.

The plasma is the fluid portion of the blood. It contains more than one hundred constituents. The most abundant constituent is water. Other constituents are proteins, carbohydrates, fats, inorganic salts, gases, waste products, enzymes, immune bodies, hormones, etc. The proteins are fibrinogen, prothrombin, serum albumin and serum globulin. The carbohydrate is glucose. The fats are neutral fats, cholesterol esters, and phospholipids. The inorganic constituents include the chlorides, carbonates, phosphates and sulfates of sodium, potassium, calcium and magnesium. The gases present in plasma are oxygen, carbon dioxide and nitrogen. The waste products include urea, uric acid and creatinine.

Serum is the clear straw colored fluid which results when blood clots in a flask or test tube. It is plasma less fibrin.

The coagulation of blood is necessary to the preservation of life. The clot, which consists mainly of fibrin, closes the openings of the injured vessels and prevents long continued bleeding.

THEORY OF COAGULATION

It is thought that when blood is shed, large numbers of blood platelets disintigrate and release a substance called thromboplastin (also obtained from tissue juice). Thromboplastin combines with calcium and prothrombin to form thrombin. Thrombin, which is believed to be an enzyme, causes a precipitation of the fibrinogen as fibrin. The following schema illustrates the events which occur in coagulation.

Calcium + prothrombin + thromboplastin → thrombin

Thrombin + fibrinogen → fibrin

Fibrin, of course, forms the clot.

The time required for coagulation or clotting varies from two to six minutes in normal individuals. Oxylates and citrates prevent the coagulation of blood by uniting with the calcium so that the chain of events in coagulation is interrupted.

To summarize, in a general way, the blood current may be likened to a river and the cells to boats floating upon it: the red cells are the freight boats loaded with oxygen which they receive in the lungs and carry to all parts of the body; the white cells are the war ships, always on the alert for an attack by disease germs; when such an attack occurs the leucocytes hurry to the invaded point and a battle ensues in which there are killed and wounded on both sides; the blood platelets act as a group of skilled workmen to stop leaks in the river banks; the dead white cells, when in large number, constitute what is known as pus or matter. The blood serum itself not only carries nourishment to all parts of the body, but, coming back, acts as a sewer, bringing away the waste products, both liquid and gaseous.

CELLS IN THE NORMAL DIFFERENTIAL FILM

We have already gone over a little about the anatomy and physiology of the blood and have been introduced to the various types of cells. Now we shall take up the various types of cells a little more in detail with particular reference to their appearance in the film stained with Wright's stain.

It has been said with much truth that an intelligent study of the stained film, together with an estimation of hemoglobin, will yield 90 per cent of all the diagnostic information obtainable from a blood examination. The stained films furnish the best means of studying the morphology of the blood and blood parasites, and, to the experienced they give a fair idea of the amount of hemoglobin and the number of red and white cells. An oil immersion objective is required.

Erythrocytes. - Normally, the red corpuscles are acidophilic. This means that they take the acid stain, which, in Wright's stain is eosin, a red dye. However, in well stained smears the red cells are a pale yellow or pink. When not crowded together, they appear as circular, homogeneous disks, of nearly uniform size. In any normal blood, however, there may be a slight individual variation. The center of each cell is somewhat paler than the periphery (outside). Red cells are apt to be crenated, that is, wrinkled from loss of water, when the film has dried too slowly.

The depth of staining furnishes a rough guide to the amount of hemoglobin in the corpuscles. When hemoglobin is diminished, the central pale area becomes larger and paler. This condition is known as hypochromia. In pernicious anemia, on the other hand, as a result of the increased hemoglobin content, many ofthe red cells may stain deeply and lack the pale center entirely.

Lymphocytes are small, mononuclear (i.e., one nucleus) cells, without specific granules in the cytoplasm. Some of the larger lymphocytes, however, may have a few, usually 5 to 10, rounded, discrete, reddish-purple granules in the cytoplasm. The lymphocytes are about the size of a red cell or slightly larger, although their diameter is influenced to a great degree by the thickness of the film, being greatest in very thin films where the cells are flattened out. The typical lymphocyte is a cell with a single, sharply defined nucleus containing heavy blocks of chromatin, staining blue with Wright's stain, while the parachromatin stains pink, and the cytoplasm a robin's egg blue. The characteristic feature of the lymphocyte nucleus is that there is a gradual transition between the chromatin and the parachromatin, so that it is practically impossible to tell where one stops and the other begins. The nucleus is generally round, but is sometimes indented at one side. Larger lymphocytes, nearly twice the size of a red cell, with paler nuclei and more abundant cytoplasm, are frequently found, especially in the blood of children, and are difficult to distinguish from monocytes. It is believed that the larger forms are young lymphocytes which become smaller as they grow older. Lymphocytes form 20 to 50 per cent of the normal differential count.

Monocytes - Under this heading we include the two types which have long been known as large mononuclear and transitional leukocytes. They are merely different forms or ages of the same cell.

The monocyte is the largest cell of normal blood, being generally two to three times the diameter of a red cell, although smaller individuals are sometimes encountered. It contains a single nucleus, which is lobulated, deeply indented, or horsehoeshaped, or less often, rounded or oval, and which is commonly located away from the center.

The zone of the cytoplasm surrounding the nucleus is relatively wide. With Wright's Stain the characteristic feature of the nucleus is for the chromatin to be in strands. There is also a relatively sharp distinction between the chromatin and the parachromatin, which results in a less densely stained nucleus than that seen in the lymphocyte, while the cytoplasm is slate colored or has a ground glass appearance. The cytoplasm sometimes appears dusted, uniformly or in patches, with fine reddish granules which are much less distinct than the granules of neutrophiles, and smaller than those occasionally seen in lymphocytes. The size of the cell, the width of the zone of cytoplasm, and the depth of color (greater in lymphocytes) of the mucleus, are points to be considered in distinguishing between those monocytes which have a round nucleus and lymphocytes, but it must be remembered that the thickness of the film has a marked influence upon the apparent size of all leukocytes. They appear larger and paler when flattened out in very thin films. Also, another differential point is that the chromatin of the lymphocyte nucleus appears to be in larger blocks while that of the moncyte nucleus tends to be in strands. The monocytes constitute, normally, from three to eight per cent of the differential count.

Neutrophils - There is usually no difficulty in recognizing these cells. Their average diameter is slightly larger than that of a lymphocyte. The nucleus stains rather deeply, and is very irregular, often assuming shapes comparable to letters of the alphabet, E,Z,S, and so forth. Frequently there appears to be several nuclei, hence the widely used name, "polynuclear leukocyte". Upon careful inspection, however, delicate nuclear bands connecting the parts can usually be seen. The cytoplasm is relatively abundant and contains great numbers of fine, neutrophilic granules. With Wright's Stain the chromatin of the nucleus is purple and the cytoplasmic granules are lilac, while in the well-stained preparation the cytoplasm itself is light pink or acidophilic.

In infections and inflammatory conditions, notably in pneumonia and appendicitis, a comparison of the percentage of neutrophils with the total leukocyte count yields more information than a consideration of either alone. In a general way, as was pointed out by Sondern, the percentage represents the severity of the infection or, more correctly, the degree of toxic absorption: while the total count indicates the patient's power of resistance. With moderate infection and good resisting powers the leukocyte count and the percentage of neutrophiles are increased proportionately. When the neutrophilic percentage is increased to a notably greater extent than is the total number of leukocytes, no matter how low the count, either very poor resistance or a very severe infection may be inferred. The neutrophils constitute 50-75 per cent of the differential count.

Eosinophils - The structure of these cells is similar to that of a neutrophils, with the striking difference that, instead of fine neutrophilic granules, their cytoplasm contains coarse, round or oval granules having a strong affinity for acid stains. They are easily recognized by the size and color of the granules which stain bright red. Their cytoplasm has generally a faint, sky-blue tinge, and the nucleus stains somewhat less deeply than that of the neutrophil. The eosinophils constitute 1-4 per cent of the differential count.

Basophils - In general, these resemble neutrophils, except that the nucleus is less irregular (usually merely indented or slightly lobulated) and the granules are larger and have a strong affinity for basic stains. They are easily recognized by the large, round or oval, dark blue or deep purple granules. The nucleus is much paler and often nearly or quite hidden by the granules. These constitute 0.25 - 1 per cent of the differential count.

Platelets - With Wright's stain they appear as spheric or ovoid, reddish to violet, granular bodies, about half the size of a red cell. The granules in the platelets usually are dark blue or purple. In ordinary blood smears they are usually clumped in masses. A single platelet lying on a red cell may be easily mistaken for a malarial parasite.

Platelets are not counted in the differential count, but it is a good thing to notice them and become familiar with the normal relative number in the blood film, so that marked decreases will be readily noticed. The normal platelet count is 500,000-1,500,000 per cu mm., with an average normal of 800,000 per cu. mm.

NOTE: This figure varies somewhat, depending on the method of counting used. The figures given here are those for the method given on page 30.

COLLECTION OF BLOOD COUNTS

Read the slip carefully - avoid collecting counts not ordered; as for example, no red count is wanted when a WBC is ordered.

Blood counts are ordered as follows:
CBC means a complete blood count; red, white, hemoglobin and two blood smears;
RBC & Hg - a red count and hemoglobin;
WBC - a white count and two blood slides;
Leukocyte and differential - the same as a WBC.

COLLECTION OF THE COUNT

(1) Always be certain you are sticking the right patient.

(2) Get apparatus ready before sticking patient.

(3) Sterilize the needle before sticking patient.

(4) Wash patient's finger with alcohol.

(5) Make a quick but deep stick into the finger.

(6) Wipe away the first drop of blood because it is mixed with alcohol and makes the count inaccurate.

(7) Moderate pressure on the finger should bring enough blood to collect the various counts. Do not press too hard as extreme pressure causes tissue juice to become mixed with blood. If necessary stick the patient again.

(8) It is best to make the blood smear first because some tissue juice tends to come with the first few drops of blood and make the red and white counts inaccurate.

(9) The red, white and hemoglobin may be collected in any order. There is no special reason for collecting a white before a red or vice versa.

(10) For the white count - draw the blood to the .5 mark on a white pipette and dilute to the 11 mark with 3% Acetic acid.

(11) For the red count - to the .5 mark on a red pipette and dilute to the 101 mark with Hayems fluid.

(12a) Talquist.

(12b) For hemoglobin - draw the blood to the 10 mark on a hemoglobin pipette and dilute to the mark with Sahli. If the patient's blood is very pale, draw it to the 20 mark.

(13) Always write the patient's name and date on the blood smears before you leave the bedside of the patient.

(14) Wrap the slip around the pipettes and blood smears and fasten with rubber band. This is especially important, if you are collecting several counts so as to avoid the error of getting counts mixed.

(15) If you have collected a count containing hemoglobin, always write the time of collection on the slip when you come into the laboratory, as hemoglobin estimation must stand 30 minutes before they can be read in the colorimeter.

SOLUTIONS IN HEMOTOLOGY

1. Hayems - for RBC

   5 grms of Mercury Bichloride
   10 grms of Sodium Chloride
   50 grms of Sodium Sulfate
   2 liters of distilled water

2. Acetic Acid 3% for RBC

   30 cc. Glacial Acetic Acid
   970 cc. distilled H_2O

3. Sahli - for hemoglobin

   10 cc. hcl to 990 cc.
   distilled H_2O

THE ERYTHROCYTE COUNT - R.B.C.

The Hayems solution preserves all the cells, so that both red and white cells are counted but as there is only about one white cell to 1000 red cells, this does not make the count so inaccurate. The total number of red cells counted is multiplied by a factor to make up for the dilution and for the fraction counted.

1. Shake the pipette vigorously for one minute to insure an even distribution of cells. This is especially important in red cell counts.

2. Blow out the contents of the stem of the pipette and fill the counting chamber by holding the pipette at the margin of the cover slip and letting a drop run under. Avoid letting the fluid run into the grooves of the chamber.

3. Count red cells under high dry lens.

4. Count 5 of the squares, one at each corner and the one in the center. There should not be difference of more than 10 in any of the squares counted. Calculate the red cells by adding 4 ciphers (0000) to the total counted.

Note: Corpuscles which touch the lower and right sides should be counted as if within the squares, those touching the upper and left sides, as outside.

TECHNIQUE OF BLOOD COUNTING - W.B.C.

The leukocyte count - The acetic acid used in diluting the blood for the leukocyte count dissolves the red cells and leaves the white cells to be counted. This solution is placed in a hemocytometer, or counting chamber and counted under the microscope. The total number is multiplied by a factor to make up for the dilution and for the fraction counted in the chamber.

1. Shake the white pipette vigorously for about one minute to insure therough distribution of the cells.

2. Fill the counting chamber by holding the pipette against the edge of the coverslip and letting enough fluid run under to just cover the rulings on the chamber. Avoid letting the fluid run into the grooves of the chamber. Always expel a few drops of the fluid before filling the chamber because the solution in the stem of the pipette does not have the accurate dilutions as that in the bulb.

3. Count the white cells under low power lens.

4. Count the cells in the 4 outer corners of the chamber and multiply the total number by 50. There should not be a difference of more than 10 in any of the 4 squares counted. If this happens refill the counting chamber and make another tabulation.

SOURCES OF ERROR

The most common sources of error in making a blood count are:

(a) Inaccurate dilution, usually from faulty technic, occasionally from inaccurately graduated pipets. Only an instrument of standard make can be relied upon, and it is best to purchase one which has been tested by the United States Bureau of Standards.

(b) To slow manipulation, allowing a little of the blood to coagulate and remain in the capillary portion of the pipet.

(c) Inaccuracy in depth of counting chamber usually due to imperfect application of the cover glass, but sometimes to faulty manufacture or to softening of the cement by alcohol or heat. A cemented slide should not be cleaned with alcohol or left to lie in the warm sunshine.

(d) Uneven distribution of the corpuscles. This results when the blood has partially coagulated, when it is not thoroughly mixed with the diluting fluid.

(e) The presence of yeasts, which may be mistaken for corpuscles, in the diluting fluid.

COLOR INDEX

This means the amount of hemoglobin in the average red blood cell of the patient compared with the normal amount.

$$\text{Color index} \quad \frac{\text{Hemoglobin per cent}}{\text{number of red blood cells per cent}}$$

Considering 5,000,000 per cu. mm. of red blood cells as 100%, the percentage of normal may be obtained by multiplying the first two figures of the red count by two.

Example

1. Red blood cells 5,000,000
2. Hemoglobin 100%

Color index $\frac{100}{50x2} \pm 1$

A normal color index ranges from 0.85 to 1.15.

MAKING AND STAINING BLOOD FILMS OR SMEARS

SPREADING THE FILM - Properly spread films are essential to accurate work. They more than compensate for the time spent in learning to make them. There are certain requisites for success with any method. (a) The slides and covers must be perfectly clean; new slides should be soaked in a 10 per cent solution of acetic acid, and then rinsed in clean distilled water, and dried; old slides are cleaned by thoroughly washing with soap and water, rubbinb with alcohol and drying on a clean towel; (b) the drop of blood must not be too large; (c) the work must be done quickly, before coagulation begins.

The blood is obtained from the finger tip, as for a blood count; only a very small drop is required, usually about twice the size of a pinhead. The size of the drop largely determines the thickness of the film. The proper thickness will depend upon the purpose for which the film is made. For the structure of blood cells and the malarial parasite it should be so thin that throughout the greater part of the film, the red corpuscles lie in a single layer, close together but not overlapping. For routine differential counting of leukocytes a film in which the red cells are piled up somewhat is best because the leukocytes are more evenly distributed, and because the number of leukocytes in a given area is greatly increased and the tedium of counting is correspondingly lessened. The film must not, upon the other hand, be so thick that identification of the various leukocytes becomes difficult. In some cases of severe anemia it is very difficult to make good films owing to the large proportion of plasma, which leads to slow drying, with consequent distortion of the red cells and the appearance of artifacts. To overcome this the films should be made very thin and dried quickly over a low flame.

WRIGHT'S STAIN

1. 0.1 grm Wright's stain. - Eosin-red-acid dye
   Methylene Blue - Basic dye
2. .60 cc. Methyl Alcohol.

THE DIFFERENTIAL COUNT - STAINING OF THE BLOOD SMEAR

1. Cover the slide with Wright's stain and let stand for 3 or 4 minutes (depending on the age of the stain, the older the stain, the less time required.)

2. Add about half the amount of buffer solution as you did the stain. Leave buffer on for the same length of time as the stain.

3. Wash the slide with distilled H_2O, remove excess stain from the back of the slide and let dry.

In case of over-staining, pour a few drops of methyl alcohol on the slide and wash with distilled water.

EXAMINATION OF THE STAINED SMEAR

1. Place a drop of cedar oil on the slide and examine with the oil immersion lens.

2. Pick out an area with good distribution and staining of the cells.

3. Using the mechanical stage work from the top of the slide to the bottom in a straight line then move the slide to one side with the other adjustment the width of one field and then work up to the top thus:

until 100 white cells have been counted and classified.

4. In order to make it easier to count exactly 100 w.b.c. the following scheme should be used. Make a rough chart with 5 horizontal columns and 10 vertical columns. The tally of each kind of w.b.c. seen is kept in the appropriate horizontal column. If you are careful not to put more than 10 tallies in any one <u>vertical</u> column, by the time you have 10 tallies in the 10th vertical column, you will have counted 100 cells. See example below:

Neutrophils	卌 I	IIII	卌	卌 I	IIII	卌	卌	IIII	卌 II	卌 卌	56%
Lymphocytes	III	卌	III	III	IIII	卌	II	卌 I	III		34%
Monocytes	I		II	I	I		II				7%
Eosinophils		I					I				2%
Basophils					I						1%
											100%

THE SCHILLING COUNT

The Schilling count is a special kind of a differential leukocyte count where, in addition to classifying the five types of leukocytes, the immature cells of the myeloblastic series are also recorded. In cases with leukocytosis the Schilling count tells the doctor the relative maturity or immaturity of the leukocytes. See the following chart.

Myeloblast	Nucleoli Cytoplasm blue, No granules
Premyelocyte	Nucleoli gone Few granules in cytoplasm
Myelocyte	Nucleus still round Many granules in cytoplasm
Juvenile	Slightly indented Nucleus
Band	Deep Indentation
Segmenter	Two lobed Nucleus
Segmenter	Three lobed
Segmenter	Four lobed
Segmenter	Five or more lobes

EXAMINATION OF RED CELLS ON THE BLOOD SMEAR

This slide is stained the same as for the differential count. Red cells are examined for:

1. Hypochromasia-paleness in the center of the cells.
2. Poikilocytosis-irregularity in shape.
3. Anisocytosis-irregularity in size.
4. Basophilic stippling-small bluish-black dots on the red cells.
5. Poly-chromatophilia-slate grey color of the cells.
6. Appearance of immature red cells-erythroblast, pronormoblast and normoblasts.

Erythroblast - stem cell of red series; large cell with a blue-grey mottled nucleus and deep blue cytoplasm.

Pronormoblast - cell size a little smaller, nucleus more compact, cytoplasm relatively larger and brownish-blue.

Normoblast - cell size, same as mature erythrocyte; nucleus small, dark and compact, cytoplasm pink, same as mature erythrocyte

Nucleated red cells are premature cells thrown into the blood stream to replace used up erythrocytes. They are most abundant in myelogenous leukemia and severe secondary anemia.

ERYTHROPOIESIS

Erythropoiesis or red cell production takes place in the bone marrow. At birth all the bone marrow of the body is active. As the person grows older, there is a change to inactive fatty bone marrow. The active bone marrow of the adult is limited to the vertebra, bones of the skull, ribs, sternum and innominate or hip bones. Blood vessels in the bone marrow run longitudinally until they split into branches and run vertically. These branches are called blood sinuses. Red cell formation takes place within the inter-sinusoidal capillaries or sinuses. Conditions favorable to red cell formation are low oxygen supply and reduced circulation. Endothelial cells line the sinuses. These cells swell and divide. If the mitotic spindle is in one direction, the cells form two more endothelial cells; if in the other direction it forms the next stage, the megaloblast - young erythroblast - normoblast reticulocyte - normal red cell.

CLOTTING TIME

1. Cleanse patient's finger with alcohol and stick.
2. Discard the first drop of blood.
3. Hold a small capillary pipette against the finger so that a drop of blood flows into the capillary pipette.

4. Note the time the sample of blood was taken.

5. Break off small bits of the pipette at intervals of 30 seconds until a thread of fibrin can be seen. Note the time this occurs. The clotting time is figured by the length of time from the moment the pipette is filled to the time the thread forms.

FACTORS IN CLOTTING TIME
(Baucroft & Quick)

Prothrombin, platelets (which release thrombokinase) calcium and fibrinogen are the blood clotting factors in normal blood. Prothrombin plus calcium plus thrombokinase equals thrombin. Fibrinogen plus thrombin equals fibrin which is the substance which produces the clot.

BLEEDING TIME

1. Cleanse the lobe of the patient's ear with alcohol and puncture so that blood will flow without pressure.

2. Start time when blood begins to appear; 30 seconds from that time, blot up all the blood with filter paper and repeat every 30 seconds, until bleeding stops.

3. Figure the time by counting the blots on the paper and divide by two, which gives the time in minutes.

MALARIAL SMEARS
Collection of Smear - "Thick Drop"

1. Place several large drops of blood on a slide.

2. With the corner of another slide, spread the blood in a circular motion, apply rather firm pressure in spreading the blood so as to bring the parasites out.

3. Let stain for 30 minutes.

Examination of Stained Smear

1. Examine under oil immersion lens.

2. Parasites appear as red dots with blue rings. The red cells have been dissolved leaving the parasites on the slides.

3. Never report a malarial smear as "positive" unless you can find at least 3 parasites on the thick drop.

Platelet Count:

THE BRILLIANT CRESYL BLUE MOIST PREPARATION METHOD. A saturated solution of brilliant cresyl blue in 95% alcohol is smeared on a clean slide and allowed to dry. The dried stain on slides keeps well and a number of slide preparations can be made at the same time and stored until needed. Before use the film of dye is gently polished with lens paper. A small drop of blood from a skin puncture wound is collected on a clean coverslip. The coverslip is immediately dropped onto the stain and the drop allowed to spread without pressure except for the weight of the coverslip. If pressure is required to make the blood spread the preparation is not suitable for platelet counts. The drop of blood should be small so that the margins extending outward do not touch more than two of the coverslip edges. The edges of the coverslip are rimmed with vaseline. After waiting at least 10 minutes for the cells and platelets to become stained, 1,000 red blood cells are counted and the platelets in the same fields noted. The count is made under oil immersion, using a bright light. A tally counter and a disc in the eyepiece to reduce the size of the field are of aid in counting. The platelets appear as spherical or vesicular bodies, blue with darker bluish granules, 1/4 to 1/2 the size of red blood cells and sometimes larger. The number of platelets is estimated from the red cell count taken at the same time the moist preparation is made and is computed according to the following equation.

Number of platelets: 1,000 r.b.c. x: Number of r.b.c. per cu. mm.

Example:

Red cells counted	1,000
Number of platelets counted	72
Red cell count	4,300,000

$$\frac{4,300,000}{1} \times \frac{72}{1,000} = \text{no. platelets per cu. mm.}$$

The normal number of platelets with the brilliant cresyl blue moist preparation varies from 500,000 to 1,5000,000 per cu. mm., with the average around 800,000. The higher count obtained with this method than with other commonly used methods is due to the fact that there is a minimum of manipulation, the platelets are not destroyed in such large numbers and the platelet fragments remain visible. Since the platelets are themselves fragments any method which preserves the fragments best is a superior method. The main sources of error are the unevenness of distribution, the small number of red blood cells counted and failure to make a satisfactory preparation. The most common errors in technic are the use of glassware that is not scrupulously clean, the use of too large a drop of blood, insufficient stain and improper light.

Reticulocyte Count:

Slides prepared with brilliant cresyl blue as for the platelet count above, may be used. The same procedure is followed in making the preparation. One thousand red cells are counted and the number of reticulocytes noted. The normal number is 0.1 to 1.0 per cent or 1 to 10 per thousand.

Results are reported in per cent.

SEDIMENTATION RATE

There are so many methods now in use for performing this test that results have not been comparable. Only one method will be given here with the normals for this method only.

This test is useful in determing the severity of the following diseases: Tuberculosis, Rheumatic Fever and Salpingitis.

Materials

1. Veinpuncture equipment.
2. Cutler tube, this must be purchased from a supply house, it is a tube graduated at 1 cc. capacity and marked into fifty 1 mm. divisions with 0 at the top.
3. 2 cc. sterile syringe.
4. 3% Sodium Citrate solution (sterile)

Procedure

1. Draw 0.1 cc. of 3% Sodium Citrate into the 2 cc. syringe.
2. Draw into the same syringe 0.9 cc. of blood from the vein.
3. Mix and pour into the upright tube.
4. Read the height of the blood cell column every 5 minutes, for one hour, plot results as a graph.

The normal for men is under 8 mm. and for women under 10 mm. in one hour, with a horizontal line.

1. Diagonal line with a fall greater than normal indicates a mild condition.
2. Diagonal curve with the fall continued in the last half hour indicates an active condition.
3. Vertical curve with the entire fall in the first half-hour indicates a more severe condition.

In an effort to use materials normally on hand in the laboratory the modified Cutler method is suggested. Select test tubes from 75 x 10 mm. stock of such caliber that 4 cc. gives a column 50 mm. high, etch the tube at this point. Put exactly 0.4 cc. of 3% Sodium Citrate in the tube. Fill to the 50 mm. mark with blood. Mix by inverting, avoid air bubbles. Set the tube in the vertical position. Measure with a millimeter ruler and record at 5,10,15,30 minutes and 1 hour. Chart on a graph.

Normal for men 2-8 mm. in 1 hour; for women 2-10 mm.

VEINPUNCTURE

For Blood Culture

Materials

1. Luer syringe, 10 cc., sterile
2. Needle, 20 gauge, sterile
3. Flask of appropriate culture medium
4. Tincture of iodine
5. Alcohol, 70%
6. Tourniquet
7. Cotton or gauze pledgets, sterile
8. Alcohol lamp if no gas burner is available

Procedure

1. Thoroughly cleanse skin over the vein and surrounding area of the arm for about three inches, with alcohol.
2. Paint over the vein with iodine and leave on for 2 or 3 minutes.
3. Light alcohol lamp or burner.
4. Unwrap syringe and insert plunger into barrel. Do not touch inside of barrel or shaft of plunger.
5. Remove plug from needle tube and flame mouth of tube.
6. Insert neck of syringe into mouth of tube and tilt tube so that needle will slide down over the neck.
7. Remove syringe and needle and set the needle firmly on the neck, being careful to touch only the hub of the needle.
8. Flame both the needle point and the mouth of the tube that contained the needle. Cover the needle with the flamed tube and set aside while completing the preparation of the arm.
9. Apply tourniquet above the elbow, not too tightly. If the vein does not distend well, have patient clench fist.
10. Sponge off the iodine with alcohol.
11. Puncture the skin with needle a little to one side of the vein and parallel to it; then enter the vein from that side about half an inch above the skin puncture.
12. After securing the desired amount of blood, loosen the tourniquet and have patient open fist.
13. Press an alcohol-soaked pledget firmly over the puncture and withdraw needle quickly. Have patient flex elbow tightly to hold pledget in place.
14. Open flask and flame mouth thoroughly, holding the syringe near but not in the flame at the same time.
15. Insert needle into flask and force blood directly into culture medium without touching sides of flask with either needle or blood. Flame neck of flask again, replug and incubate.

For Other Purposes

In taking the large number of routine blood samples required for other purposes which do not demand sterile blood, it is simpler to use only the sterile needle, as so many syringes are seldom available. The method is simple and takes little time, provided directions are followed and a little patience is exercised.

Procedure

1. Swab the site with iodine, followed by alcohol or acetone. Acetone alone may be used.
2. Tighten tourniquet about the upper arm enough to dilate the vein firmly.
3. Remove the needle from its tube and take the stylet out, being careful to touch only the hub.
4. Hold the needle tightly between the thumb and index finger at the hub. Insert the tube for the blood specimen below the needle, grasping it with the third and fourth fingers, so that the hub of the needle is just within the mouth of the tube. This is much easier to accomplish if the patient's arm is allowed to hang straight down.
5. Puncture the skin a little to one side of the vein and parallel to it. If the needle is sharp, this can be done with one quick motion and is not at all painful.
6. Turn the needle point slightly toward the vein and enter with a quick, short stab. If you turn the point too squarely toward the vein you risk puncturing both walls.
7. When you have collected 10 to 15 cc. of blood, loosen the tourniquet.
8. Press a pledget of cotton soaked in alcohol or acetone over the puncture and withdraw the needle quickly, maintaining the pressure until the bleeding, if any, has stopped.

Care of Needles and Syringes

1. As soon as the blood sample is taken, shake as much of the blood from the needle as possible and drop it into a beaker of water to lake the blood. On return to the laboratory, clean thoroughly with cold water and dry by forcing alcohol followed by ether through the bore. Never put a wet needle away, as rust is dangerous.

2. Replace the stylet, leaving the loop of wire outside the point for the protection of the latter. Slide the needle, point down, into a Watterman tube, plug tube with cotton and sterilize by dry heat or in the autoclave.

3. Sharpening the needles is best done on the finest grade of emery cloth stretched on a flat surface. Finish on a fine blue water stone. Even the finest grade of emery or carborundum, if used alone will leave a slight raw edge that may cause too much pain.

4. Syringes must never be left with the plunger in the barrel after use, no matter what has been in the syringe. Always wash out the syringe with water immediately after use and leave the plunger out until both it and the barrel have been carefully dried. Once a plunger has been "frozen" in the barrel, it may not be possible to remove it. Often the best way to free it is to force cold water through the neck of the barrel against the head of the plunger, using another syringe with a needle small enough to insert into the neck. Warming the barrel in hot water may loosen it. Soaking in cold water for several days may be necessary. Never use force.

5. To sterilize, wrap the plunger and barrel separately in gauze, with an outer wrapping of heavy paper. Secure the wrapping with a turn or two of ordinary twine, tied in a slip knot to expedite unwrapping.

Cautions

1. Certain dangers and discomforts to the patient must be avoided. These are (a) Infection, (b) Injury to the vein wall, (c) Hematoma, (d) Needless pain.

2. Infection is due to carelessness. Be certain your needles and syringes are sterile. Never touch the shaft of the needle to anything that is not sterile before taking the blood.

3. Injury to the vein wall may cause a clot to form on the wall. This may break free in the blood stream and death may result. Causes: Dull or rusty needles, too much movement of the needle point while in the vein, passing of the stylet through the needle while still in the vein in the attempt to free the needle from clots. Never pass the stylet through the needle before withdrawal. If clots plug the needle, withdraw it and try the other arm.

4. Hematoma (blood tumor) is often very painful and may become infected. Causes: Too large a needle in a delicate vein, withdrawal of the needle before tourniquet is loosened, making insufficient pressure over the puncture after withdrawal.

5. Needless pain is often due to excess of care and slowness in making the puncture. Dull needles most often cause it. A sharp 17 gauge causes less pain than a dull 20 gauge. Remember that those ill enough to be in a hospital may be greatly set back by even a slight painful shock, especially if many blood tests have to be taken. It is always better to make patients lie down or sit in comfort while blood is being taken.

BLOOD TYPING

The typing and cross-matching of human blood for transfusions is the most exacting procedure which you as laboratory technicians will be called upon to do. A mistake, or perhaps just a little carelessness, in carrying out this procedure, can very easily cause the death of a patient. As the procedure of blood typing and cross-matching, and the theory behind it, becomes clear to you, you can readily see the truth of this statement.

Before plunging into the procedure itself, there are a few fundamental principles which it is necessary to know. First, what is agglutination? Agglutination means sticking together. It is the process of being clumped together. In the logging business up north logs are cut and thrown into rivers and are floated downstream to saw mills. Now as long as these logs remain separate, they float merrily down the stream, but, once they become jammed or agglutinated, the logs can no longer get through the narrow places and all are held up. So, in the human blood vessels, if the red cells become agglutinated, the cells become jammed in the narrow vessels, and the unfortunate person dies. Placing the wrong type of blood in a patient's veins by transfusion may cause such a tragedy. Another factor to be considered in the transfusing of blood is hemolysis. Hemolysis is the process by which the red blood cells are broken up or dissolved so that they give up their hemoglobin to the plasma. Hemolyzed blood is bright cherry red and does not have adequate oxygen carrying power. Hemolysis of blood in the body also will cause death and again transfusion with the wrong type of blood will cause hemolysis. However, since hemolysis does not occur without agglutination, it is sufficient to test for agglutination only.

There are two factors which are necessary to cause agglutination. A factor called an agglutinin contained in the serum and a factor called agglutinogen, contained within the red cells. Whenever the two like factors come together, agglutination takes place. For example, a blood having agglutinin (a) in the serum will cause agglutination when it comes in contact with red cells having agglutinogen (A). For convenience the agglutinogens are designated by a capital letter and the agglutinin with a small letter.

There are four main types or classes of human blood which we shall classify according to the method of Landsteiner in which the blood type is designated by the agglutinogen contained in its red cells, namely, AB, A, B, and O. The last type is called O since there are no agglutinogens in its cells. See figure below:

Type Agglutinogen		Agglutinin		Moss	Jansky	Incidence
AB)		o)		I	IV	3%
A)	Rbc	b)	serum	II	II	42%
B)		a		III	III	10%
O)		ab		IV	I	45%

From the above figure, it can be seen that each type has an opposite kind of agglutinin in the serum, so that the blood does not become agglutinated within itself.

If you look at the figure you can see that type AB cannot be given to another type except its own because in the serum of the other three types, there is a corresponding agglutinin designated by a small letter which will cause agglutination of the blood. Likewise, type A may not be given to B or O, because of the corresponding agglutinins in the serum and type B may not be given to A or O.

Again looking at the figure you can see that if this is so then type AB may take blood from any of the other three types because there are no agglutinins in the serum. That is true and for this reason type AB is called the Universal Recipient, but, the Serum from anyone of the other three bloods contains agglutinins which will agglutinate the cells of the recipient (or patient). However, in an emergency, if type AB donor cannot be found and if the blood is given very slowly, any of the three other types of blood may be given to a type AB recipient since the volume of serum of the blood of the donor is so small as compared to that of the recipient, that the foreign agglutinins are rapidly diluted beyond doing any harm.

Likewise, a study of the figure will reveal that type O blood could be given to any of the other three types since it contains no agglutinogens in the cells. That is true and for this reason type O blood is called the Universal Donor. Again the same reasoning holds true. Type O blood may be given to or used as a donor for any of the other three types in an emergency, if given slowly and with care. Again the donors serum contains agglutinins which will agglutinate the cells of all other types but the donors serum is considered to be so diluted by the greater volume of serum of the recipient as to make transfusion possible, if done slowly and carefully.

Before I go further, it may be well to explain that blood transfusion is the process of introducing blood from a donor into a vein of a recipient.

To avoid any unwanted reactions, which might prove disasterous, transfusions are always given with blood of the same type when possible.

Although there are only four main types of human blood, there are certain subtypes which often give bad reactions. For this reason, it is necessary to cross-match the blood of the recipient with that of the donor in addition to having the same type of blood. There are two kinds of cross-matching - a major and a minor. The major cross-match is a comparison of the donor's cells with the serum of the recipient while the minor cross-match is a comparison of the recipient's cells with the serum of the donor. Each time a transfusion is given a major and a minor cross match is done just before the operation regardless of whether the donor has given the recipient blood before or not. This eliminates any possibility of a mistake.

Method

A. Materials needed
   1. Wassermann tubes - 1 for each.
   2. Vein puncture needle - sterile, 1 for each.
   3. Small pipetes or medicine droppers.
   4. Solution of 0.85% saline or 2% sodium citrate in 0.85% saline.
   5. Known type A and type B serum.

B. Procedure.

1. First make a list of the donors giving each a number, then number the tubes accordingly. Then place 1 cc. of saline in each of the numbered tubes.
2. Puncture the finger of the first man on the list and place one or two drops of blood in test tube number one; then one or two drops of blood from second man in tube number two, etc. This gives cell suspensions in numbered tubes corresponding to the numbers of the men on the list.
3. On a clean glass slide make two large rings with a china marking pencil. Mark the first ring A and the second ring B. Number the slide to correspond to the number of the donor. Then place one drop each of Type A and B serum on their respective rings. Then place one drop of the cell suspension on each drop of serum and tilt the slide in a rotary motion to mix the cells and serum. Then cover with a petri dish (to avoid evaporation) and allow to stand for 20 minutes. Then read with the low power objective of the microscope. Agglutination in A only means type B. Agglutination in B only means type A. Agglutination in A and B means type AB. Agglutination in neither A or B means type O.
4. Procedure for cross-matching. - blood from a vein is needed from both the donor and the recipient. Draw about 8 cc. of blood from a vein of the donor and from the recipient. Place each in a dry tube. Then by dipping the butt end of the needle into a tube containing 1 cc of saline, a cell suspension can be made from each.
5. Allow the specimens of whole blood to stand for 10-15 minutes until a clot is formed. Then carefully slip a wooden applicator stick between the side of the tube and the clot to separate it. Then centrifuge to separate the clear serum. The serum can then be used for both serological tests and cross-matching.
6. Cross-matching - again make two rings on a slide. Mark the first R and the second D and number the slide to correspond with the number of the donor. In R place one drop of the recipient's serum and in D place one drop of the donor's serum. Then to R add one drop of the donor's cell suspension and to D add one drop of the recipient's cells. Then cover and read in 20 minutes as before. Agglutination in either R or D or both, means the donor is not compatible and cannot be used. Of course, if there is no agglutination in either R or D the donor is compatible and may be used.

NOTE: "R" is the major cross-match

"D" is the minor.

The New Method of Blood Typing, Using The International (Landsteiner) Classification and The New Powdered Rabbit Anti Serum

1. As it has been found to be impractical to supply human sera for performing these tests, especially prepared dried rabbit sera will be used. The two reagents required consist of mixtures of sucrose and dried sera from rabbits which have been immunized with human erythrocytes of groups A and B respectively. Such sera are standardized and tested by the manufacturer, and when used as recommended will cause prompt macroscopic agglutination (usually within 1/2 minute) of the respective types of human erythrocytes. Medical Department purchase specifications require of the manufacturer that the potency of serum be such that when mixed according to directions issued by the manufacturer specific macroscopic agglutination A, B, and AB human red blood cells respectively will occur in a period not to exceed one minute. (To avoid occasional errors they should be rechecked after 10 min.)

2. It should be borne in mind that when such sera from rabbits immunized with human red blood cells are used for the determination of blood groups the agglutination reaction is direct and is not reversed as when human typing sera are used. For example, when using the rabbit sera, red blood cells which agglutinate in Anti A serum belong to group A, and red blood cells which agglutinate in the Anti B serum belong to group B.

3. Technique of blood typing:

a. Materials:

Anti A, powdered serum, rabbit (colored with methylene blue)

Anti B, powdered serum, rabbit (colored with eosin)

Physiologic salt solution

Small test tubes

Microscopic slides

Wooden toothpicks or applicators

Wax pencil

b. The Test

(1) Carefully cleanse with alcohol and puncture the finger of the individual whose blood is to be typed and collect one or two drops of blood in a small test tube containing 1.0 cc. of physiologic salt solution. This makes an erythrocyte suspension of about ten percent.

(2) With a wax pencil draw a line across the middle of a clean glass slide and label one end "A" and the other "B". Using a clean medicine dropper (washed with three changes of saline between each test) place in the center of each of the two ends of the slide, "A" and "B", a large drop of the ten percent blood suspension to be tested. Then, with the small end of the blue-tipped toothpick, dip up a small mound (about 2mm. long) of group A powdered serum (colored with methylene blue) and add this to the drop of blood suspension on the "A" end of the slide. Likewise, using the red-tipped toothpick, add a similar amount of group B powdered serum (colored with eosin) to the "B" drop of blood. Mix the preparations thoroughly by stirring each with the unused end of a clean new wooden toothpick. Allow the preparations to stand one minute and read the results.

4. Interpretation of results:

a. If no agglutination occurs either in serum A or B, the blood being tested belongs to group O (universal donor).

b. If agglutination occurs in the group A serum only, the blood belongs to group A.

c. If agglutination occurs in the group B serum only, the blood belongs to group B.

d. If agglutination occurs in both the A and B area, the blood belongs to group AB.

Note: It will be noticed that the Anti A serum imparts to the saline suspension of blood cells a bluish green color, while the Anti B serum gives an eosin red color. This should prevent confusion in reading the results of the test, as the colors remain constant, and thus errors in the labeling of slides should be eliminated.

5. A Suggested Procedure for Determining and Recording the Blood Types of Military Personnel.

The test to be used in determining the blood types of military personnel is so simple that elaborate laboratory facilities will not be required. In small posts or isolated detachments the tests can be done by the surgeon with a few enlisted assistants. In large organizations it is suggested that they may be performed expeditiously by using one or more "blood-typing teams" composed of Medical Department personnel as outlined below. The individuals to be typed should be required to report for the examination at some convenient central place and to bring with them their identification tags. Each "blood-typing team" should be supplied with a Graphotype machine for recording the results of the tests on the tags. It is estimated that a single team should be able to determine the blood types and record the findings for 400 individuals in an eight-hour day.

ORGANIZATION OF A BLOOD-TYPING TEAM

The individuals whose blood is to be typed should report by organizations to a place designated by the officer in charge of the blood-typing team, after which they will pass through the following "stations" in sequence:

STATION I At this station an enlisted man seated at a table, adding to small glass vials 1.0 cc. amounts of physiological salt solution by means of a calibrated medicine dropper, will give one tube of salt solution to each individual to be tested.

STATION 2 At this station two (2) enlisted men, taking alternate individuals, will collect from the finger of each individual four drops of blood, adding this directly to the vial of salt solution. The vial will be returned to the individual who will carry it to station 3.

STATION 3 At this station two (2) enlisted men, taking alternate individuals, will prepare the tests by placing with a clean* medicine dropper a drop of the blood cell suspension on each of the two labeled ends of a glass slide, and then adding the dried sera A and B respectively as outlined in paragraph 5 c above. The slide containing the individual's blood grouping test and his vial will be returned to him, and he will then carry them to Station 4 where the results will be read and recorded.

* Note: A single medicine dropper may be used continuously provided it is rinsed three times with clean physiological saline between tests. This can be done by using a beaker or glass filled with clean physiological saline and a small container in which to discard the washings. (Care should be exercised to prevent contamination of the clean saline with the blood washings.)

STATION 4 At this station one (1) enlisted man will read the result of the test to the Graphotype operator who will record the result on the individual's identification tag. The individual will then carry his vial of blood cells, his blood grouping test slide, and his identification tag to the next station.

STATION 5 Here the medical officer will verify the result of the test and check this against the record made on the identification tag in order to prevent errors in recording.

STATION 6 At this station the individual who has been examined will discard his glass vial and test slide in suitable containers provided for that purpose. Two enlisted men will wash and dry the used glassware and prepare them to be used again.

Cerebrospinal Fluid

Now we shall take up the cerebrospinal fluid with particular reference to the routine tests which you may be called upon to run on the fluid.

Before going into the various procedures, it might be well to get a little background. The brain is a mass of specialized nerve tissue, composed of white and gray matter and a special type of connective tissue called glial tissue, which is contained within the bony cranium or brain case. The chief parts of the brain are the cerebrum, the mid-brain, the cerebellum, and the brain stem, or medulla. The cerebrum is divided into two cerebral hemispheres and is the seat of intelligence, that is, the center of thought, memory and association, etc. The mid-brain is the seat of pain, touch, water balance and temperature control. The cerebellum is the seat of balance and the coordination of muscles. The medulla is where the vital centers of respiration and circulation are located. The spinal cord is a continuation of the medulla. The brain and spinal cord are covered by three continuous membranes, called the meninges; namely, the Dura Mater, the Arachnoid and the Pia Mater, going from the outside in. The Pia Mater is quite thin and is very closely applied to the surface of the brain and spinal cord and dips into all of the convolutions or wrinkles of the brain. It is between the Arachnoid and the Pia Mater that the cerebrospinal fluid lies.

The brain has four ventricles or spaces within its substances. The first two are called the <u>lateral ventricles</u> and are situated one in each cerebral hemisphere. The <u>third ventricle</u> is situated in the mid-brain and the <u>fourth ventricle</u> is situated in the medulla. All of the four ventricles are connected and the fourth ventricle is continuous with the small central canal of the spinal cord.

The cerebrospinal fluid forms a "shock absorber" for the brain and spinal cord, since they are both completely surrounded by the fluid or you might say, the brain and spinal cord float in the cerebrospinal fluid. In addition, this fluid forms a sewer into which the waste products of the brain and spinal cord are discharged.

The cerebrospinal fluid is secreted by the choroid plexuses, which are situated in the ventricles of the brain, chiefly the lateral ventricles. The fluid is more than just a filtrate of the blood since it contains normally about half the concentration of the blood sugar; traces of protein and chlorides, etc.

The cerebrospinal fluid passes out of the ventricles through small holes in the roof of the fourth ventricle to enter the subarachnoid space where it circulates slowly about the brain and spinal cord. The volume of spinal fluid varies between 100 and 150 cc. normally and has a specific gravity of from 1.003 to 1.008. The cerebrospinal fluid is absorbed into the blood stream by structures called Arachnoid Villi or Pacchionian Bodies which are situated in the Dura at the top of the brain in the mid-line above the groove between the cerebral hemispheres.

The cerebrospinal fluid for diagnostic and therapeutic purposes is withdrawn from the sub-arachnoid space in the spinal canal by means of inserting a long hollow needle, usually under local anesthesia, between the spinous processes of the third and fourth lumbar vertebra. I wish to emphasize that the needle is not introduced into the spinal cord. As a matter of fact the spinal cord ends at the first lumbar vertebra. The meninges extend down farther than the cord and form a sac which contains spinal fluid and the numerous nerves which form the cauda equina or horses tail.

Spinal fluid is normally clear like water and has a specific gravity, as was already mentioned, of 1.003 to 1.008, or slightly heavier than water. Usually not more than 10 or 15 cc. are withdrawn at one time. The specimen submitted to the technician is usually submitted in three test tubes, numbered from 1 to 3 in the order in which they are withdrawn. The first specimen may be slightly pink from contamination by blood picked up by the needle on the way in; the second specimen and surely the third should be clear, if the cerebrospinal fluid is normal. In cerebral hemorrhage or stroke, or cases of skull fracture with lacerations (tears) of the brain, all three specimens may be bloody. In cases of brain tumor with slow chronic bleeding in which the blood has become depigmented, the fluid may be straw-colored or xanthochromic. In cases of meningitis, the fluid may be cloudy.

Routine Tests

The routine tests on cerebrospinal fluid are total and differential cell counts, microscopic for bacteria, Pandy test for globulin, Colloidal Gold test, Kahn and Wasserman tests. In addition, special tests, such as specific gravity, qualitative and quantitative sugar, total protein and quantitative chloride tests may be run. It is only with the first group that we shall concern ourselves.

I. Cell Counts

A. Total cell count: this determination is done upon the clearest of the three specimens. A special counting chamber may be used but a regular hemocytometer will suffice. If all cells, both red and white, are to be counted; fill a white pipette with spinal fluid (no dilution), after first shaking the specimen, then fill the counting chamber in the usual manner and count all cells in the entire ruled area. That is, count 9 large squares and multiply the total by 10/9 which gives the total cells per cu. mm. If the leukocytes only are to be counted, first rinse out the white pipette with Glacial Acetic Acid and let the acid run out by gravity (do not blow it out) then fill the pipette with cerebrospinal fluid and count in the same way, using the same factor, 10/9. The normal count is 1 to 7, 10 is the maximum in health.

B. Differential Count: the differential count is done in the same manner as that on a blood film.

An increase in cell count, together with predominence of lymphocytes (more than 70%) strongly suggests tuberculosis meningitis or syphilitic disease of the nervous system, since it occurs in about 90 to 95% of cases. The number of cells in these cases varies but usually runs between 25 and 100 per cu. mm.

In all forms of acute meningitis, the total cell count is high, 100 to several thousand, and neutrophils predominate. A notable number of monocytes may also be present, especially in acute epidemic meningitis.

II. Microscopic for Bacteria

A. This test may be run with the first specimen of fluid. In general, the specimen is centrifuged and a smear is made from the sediment. The smear is then stained by Gram's method and examined under oil. If tuberculosis meningitis is suspected, a tube of fluid is allowed to stand overnight. In cases of tuberculosis meningitis, if the fluid is allowed to stand for 12 to 24 hours, a pellicle or cobweb like veil will form in the spinal fluid. The pellicle will entrap the tubercle bacilli, which can be found by placing the pellicle on a slide with an applicator stick and then staining the slide for tubercle bacilli by the Ziehl-Neelsen Technique.

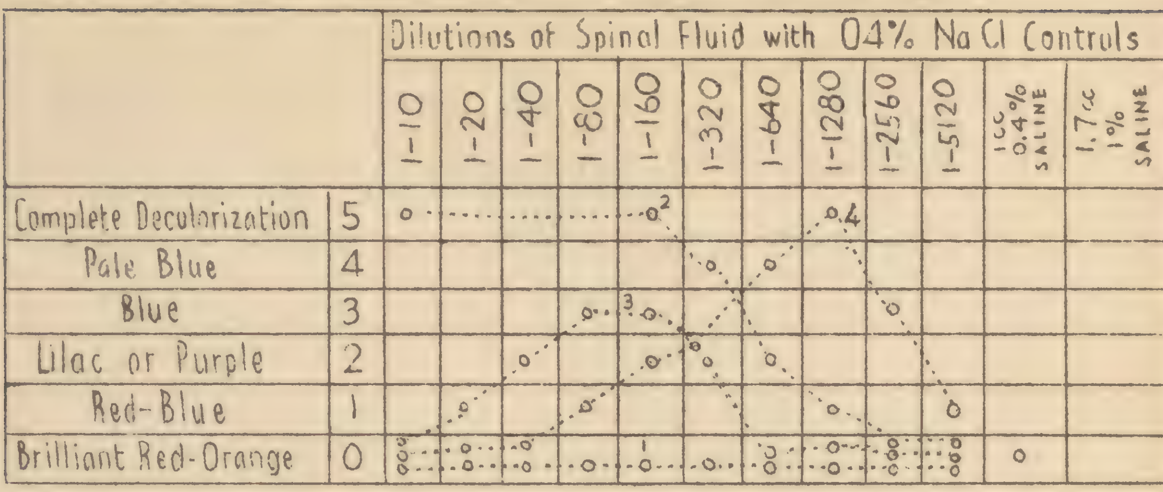
III. Pandy Test for Globulin

A. This is a simple sensitive test which should be run on the clearest of the three specimens. One small drop of spinal fluid is dropped into a small test tube containing 1 or 2 cc. of Pandy's reagent (a 10% aqueous solution of phenol crystals) a faint bluish white cloud like veil of smoke is a positive test.

IV. Colloidal Gold Test

A. Lang's Colloidal Gold Test, introduced in 1912 and now very widely used, consists in mixing cerebrospinal fluid in certain proportions with a colloidal solution of gold. Normal cerebrospinal fluid causes no change in color.. Fluids from cases of syphilis and certain pathological conditions of the nervous system, induce changes in color of the gold solution from red to purple, deep blue, pale blue, or colorless. Moreover, the dilution at which the maximum color change occurs is more or less characteristic of the different pathologic conditions.

The typical "curves"are shown in the Figure:



The test gives its most consistent and valuable results in cases of general paresis (a late effect of syphilis of the brain). In encephalitis lethargica and poliomyelitis (infantile paralysis) a typical curve in the tabetic zone has been observed. The exact explanation of the test is not yet wholly clear, but it is undoubtedly dependent upon the presence of a globulin.

V. Technic of Test - arrange a series of 12 clean test tubes in a rack. Place 1.8 cc. fresh sterile 0.4% solution of sodium chloride in the first test tube and 1 cc. in each of the others, except the twelfth. In the twelfth test tube place 1.7 cc. of sterile 1% solution of sodium chloride. To the first tube add 0.2 cc. of the spinal fluid, which must be free from any trace of blood. Mix well by sucking the fluid up into the pipette and expelling it, and then transfer 1 cc. to the second tube. Mix and transfer 1 cc. to the third tube, repeating this down the row to the tenth tube and discard the last 1 cc. portion. This leaves the eleventh and twelfth tubes, with only salt solution to serve as controls. To each of these test tubes add 5 cc. of Colloidal Gold Solution. Let stand at room temperature for an hour or longer, at the end of which time, in the case of a positive reaction, the solution in some of the tubes will have changed from red to purple, deep purple, pale blue, or colorless. In the case of normal fluids, no change will occur. The fluid in the eleventh and twelfth tubes which serve as controls, should be orange-red, and colorless, respectively. The results are usually charted, as shown in the figure, in which each column represents a tube. For the purpose of brevity, the colors may be indicated by the corresponding numbers, which are placed in the same order as the tubes. Thus the paretic curve may be expressed as 5555542100, or this may be called a first zone curve. A tabetic curve or middle zone curve may be expressed as 0123320000. A meningitic curve or last zone curve may be expressed as 0001224531.
(See Pages 92-93)

VI. Spinal Fluid Wasserman and Kahn Test: these tests may be performed on a bloody as well as a clear specimen, if the fluid is first centrifuged. As a general rule the Kahn Test is done first and if positive, is then checked with a Wasserman. As these tests, when performed upon spinal fluid, are no different from the same tests as performed on blood, they will not be mentioned here.

THE URINE

Color

1. The color of urine is subject to wide variations but possesses some diagnostic importance.

2. Normally, it is yellow or reddish-yellow (amber) due to the presence of several pigments, chiefly urochrome.

3. The color depends largely upon the concentration of urine. Dilute urines are usually pale while concentrated urines are dark. Acid urine is usually darker than alkaline urine.

4. Color may be greatly changed by abnormal pigments and by various drugs and poisons, as follows: Blood: red or brown; smoky. Bile: yellowish brown, turning green; yellow foam. Chyle: milky. Methylene blue: greenish-blue. Phenols: olive-green to brownish black, etc.

5. For uniformity in recording color, Vogel's scale is recommended, the urine being filtered and viewed by transmitted light in a glass 3 or 4 inches in diameter; pale yellow, light yellow, yellow, reddish - yellow, yellowish-red, red, brownish-red, reddish-brown and brownish-black. To these may be added greenish-yellow, olive, milky, etc.

Transparency and Sediments

1. Freshly passed urine is usually clear or transparent, but may be cloudy due to the presence of phosphates or pus. The former disappears upon the addition of acid; the latter does not, but may become gelatinous (Donne's Test). A freshly passed urine may also be cloudy with bacteria or comparatively clear with numerous shreds of mucopurulent material (chronic urethritis).

2. A record of the transparency is only of value in comparatively fresh specimens. All become cloudy with bacteria and alkaline salts upon standing as the result of decomposition.

3. Upon cooling and standing all specimens develop a faint cloud of mucus, leukocytes and epithelial cells which settle to the bottom - the so-called "nubecula." This has no significance.

4. Acid urines may develop a white or pinkish sediment of amorphous urates.

5. Alkaline urines may develop a heavy white sediment of amorphous phosphates.

6. Pus gives a heavy mucoid whitish sediment.

7. Blood gives a reddish-brown smoky sediment.

8. Bacteria give a uniform cloudiness which cannot be removed by ordinary paper filteration.

9. The following terminology is recommended:

   a. Clear, slightly cloudy, very cloudy.

   a. Sediment: slight, moderate or heavy; white, pinkish, red, brown, reddish-brown, etc., shreds present or absent.

Determination of Reaction

1. Normally fresh voided urine is acid in reaction, the PH ranging from 4.8 to 7.5 with a general average of 6. Twenty-four hour specimens are less acid than freshly passed urine and may be neutral or even slightly alkaline as a result of standing.

2. Freshly passed urine may be neutral or alkaline as the result of the administration of alkalis, retention with "ammoniacal decomposition, etc."

3. Diet influences the reaction.

Litmus Test

For ordinary purposes the reaction may be determined with good grades of blue and red litmus papers.
Blue turning red; acid
Red turning blue; alkaline
No change in either; neutral
Changes both red and blue; amphoteric

Specific Gravity

Specific weight or specific gravity denotes the weight of a body as compared with the weight of an equal bulk or equal volume of another substance, which is taken as a standard or unit. This standard adopted for all solids and liquids, if not otherwise stated, is water at a temperature of 25°C.

Determination of Specific Gravity

The normal range is from 1.015 to 1.030. Pathologically it may vary from 1.001 to 1.060. If the specimen contains but a small or average amount of sediment it makes but little difference whether the urine is mixed up or the specific gravity taken without mixing in order to use the sediment later for microscopical examination. If, however, there is a large amount of sediment the specific gravity is almost always increased by about 0.002 after thorough mixing.

For ordinary determination the Squibb urinometer may be used but the urinometer used with the immiscible balance is probably the best on the market. It settles down quickly after spinning without bobbing or swaying, and its special scale makes it much easier to read. With the Squibb urinometer the technique is as follows:

a. Fill the cylinder without producing bubbles. The specific gravity may be taken without mixing the urine.

b. Float the hydrometer so that it does not touch the bottom or sides.

c. Make the reading from the bottom of the meniscus.

Qualitative Detection of Albumin

Principles - Normal urine contains a trace of albumin which is too slight to be detected by the simple tests in general use, a large number of which have been described. All depend upon its precipitation by chemical agents or coagulation by heat. All precipitate both serum albumin and serum globulin and do not differentiate between these two proteins. Most are subject to some error largely due to the precipitation of mucin or other constituents. All require the use of clear specimens, preceded by filtration, if necessary, in order to detect small amounts of albumin. The methods here given are recommended for ordinary routine work.

Heat and Acid Test

1. Boil about 5 cc. of urine filtered if necessary, in a test tube for about a minute. Hold with a clamp or piece of filter paper folded around the neck. Boil the upper portion only.
2. Add one or two drops (no more) of concentrated nitric acid or three to five drops of 5% acetic acid solution.
3. A white cloud now disappearing is due to earthy phosphates. Effervescence is generally due to carbonates from the food.
4. A very faint trace of albumin may appear only upon the addition of the acid. Larger traces appear upon boiling and may become heavier upon the addition of the acid. The addition of too much may dissolve faint traces of albumin and give a falsely negative reaction.
5. For the routine testing of a large number of samples by this method, use numbered test tubes and place in a boiling water bath for at least five minutes.

Robert's Test

1. The test may be carried out by contact with urine in any of the following ways:
   a. Place a few cc. of the reagent in a conical glass or test tube. Tilt and run clear urine from a pipet or medicine dropper down the side to give a sharp line of contact.

   REAGENT

   Magnesium sulphate (saturated aqueous solution - 5
   Nitric acid (concentrated) - - - - - - - - - - - - 1

   b. Place urine in a horismascope and underlay with agent. This instrument is too fragile and too expensive for general use although good for office work.
   c. Or immerse a pipet in the urine, wipe off the outside, and immerse in the reagent.

2. If albumin is present, white rings appear at the line of contact, best seen against a black background at a distance of a few feet.

Methods of Recording Reactions

- = Negative

± = Very slight trace. Cloudiness or ring can just be seen against a black background.

+(1) = Slight trace. Cloudiness distinct but not granular; no definite floculation. Or the cloud or ring is sufficiently definite to be seen without a black background.

+ +(2) = Moderate trace. Cloudy distinct and granular without definite flocculation. Or the ring is dense but not wholly opaque when viewed from above.

+ + +(3) = Heavy cloud. Cloud is dense with marked flocculation or the ring is heavy.

+ + + +(4) = Very heavy cloud. Heavy precipitate to boiling solid or very dense ring.

Detection of Dextrose (glucose)

Principles -

1. Dextrose or glucose readily reduces the oxide of copper in alkaline solution. When the whitish-blue cupric hydroxide in suspension in alkaline solution is heated it is converted into insoluble black cupric oxide, but if sugar is present this is reduced to insoluble yellow or red cuprous oxide.
2. A large number of tests have been devised on this principle for the detection of sugar in the urine but that of Benedict is recommended because of its sensitiveness, simplicity and freedom from error. The qualitative reagent does not react with the normal sugar of the urine but detects increases above this level as low as 0.2 per cent. Furthermore, uric acid, creatinine, chloroform, formalin and other aldehydes do not interfere to such an extent as in the case of Fehling's test.
3. If albumin is present in large amounts, it may interfere with the precipitation of copper and should be removed by acidifying with acetic acid, boiling and filtering. Small amounts need not be removed.

BENEDICT'S TEST

1. Place 5 cc. of Benefict's qualitative reagent in a clean test tube.

BENEDICT'S QUALITATIVE REAGENT

Copper sulphate	17.3 gm.
Sodium Citrate	173.0 gm.
Sodium carbonate (anhydrous)	100.0 gm.
Distilled water to make	1000.0 cc.

Dissolve the citrate and carbonate in about 500 cc. of distilled water by boiling. Filter through paper. Dissolve the copper sulfate in about 100 cc. of water. Add the copper solution slowly to the citrate and carbonate solution and stir continuously while adding. Measure and add sufficient water to make the total volume 1000 cc. Do not use for quantitative test.

2. Add 0.5 cc. of urine (8 drops) and mix thoroughly.
3. Boil thoroughly for two and one-half to 5 minutes; or place tubes in a boiling water bath for five minutes - a particularly convenient method when conducting a large number of tests at one time.
4. Allow to cool spontaneously.
5. If no sugar is present the solution will remain clear or show only a slight turbidity of a faint bluish color due to urates. If sugar is present, a green, red or yellow precipitate will form, the color depending upon the amount of sugar present.
6. Even 0.25% glucose yields a large bulk of precipitate, filling the solution and rendering it opaque so that the test may be applied as readily in artificial light as in daylight.
7. The following scheme may be used for reporting (after Todd and Sanford).

+ (1) = Slight trace. No reduction is evident during boiling but appears upon cooling (greenish).

+ + (2) = Trace. Reduction occurs after about one minute's boiling. (Yellow)

+ + + (3) = Moderate. Reduction occurs after ten to fifteen second's boiling. (Orange)

+ + + + (4) = Large amount. Reduction occurs almost immediately after adding urine to the boiling reagent. (Brick red)

Haine's Test

Use three parts of Haine's Copper Solution and not more than one part urine. Boil over flame for about 1 minute. Calculate results as with Benedict's Test.

(Note) Haine's Solution, due to the presence of glycerine, seems to keep longer without deterioration than most other solutions. However, Benedict's is the more acceptable test, for the presence of sugar.

Detection of Acetone

Principles

The detection of acetone in undistilled urine is based upon a color reaction with nitroprusside (Rothera's test) in which there is a formation of ferropentacyanine with the isonitro compound of the ketone or the formation of such an ion with the isonitro-amine derivative of the ketone.

Rothera's Test

1. To 5 or 10 cc. of urine add 1 gram of ammonium sulphate.
2. Add 2 or 3 drops of freshly prepared 5% solution of sodium nitroprusside.
3. Mix thoroughly.
4. Overlay with ammonium hydroxide.
5. If acetone is present, a permanganate color will develop at the line of contact.

A modification of this test is as follows:

1. Place 2 or 3 cc. of urine in a test tube.
2. Add 2 to 5 drops glacial acetic acid.
3. Add 1 cc. nitroprusside solution (10% aqueous solution). (Or place 2 or 3 small crystals of nitropursside into the urine)
4. Overlay with ammonium hydroxide.
5. Record as above.

Detection of Diacetic Acid

Principles

The detection of diacetic acid depends upon the production of a bordeaux red or violet red color with a dilute solution of ferric chloride.

Gerhardt's Test

1. To about a half test tube full of fresh urine add a 10 per cent ferric chloride solution drop by drop until the phosphates are precipitated.
2. Filter.
3. To the filtrate add more of the ferric chloride.
4. If diacetic is present the solution will turn a Bordeaux Red color.

If doubtful apply the following test:

Lindomann's Test

1. To 4 cc. of urine in a test tube add 2 or 3 drops of glacial acetic acid, 5 drops of Lugol's solution and 1 cc. of chloroform.
2. Shake well and allow the chloroform to settle.
3. If diacetic acid is present, the chloroform does not change color, but becomes reddish-violet in its absence.
4. If the urine contains much uric acid, use double the amount of Lugol's solution.

Detection of Indican

Principles

The detection of indican by the test given below depends upon its decomposition and subsequent oxidation of the idoxyl set free into indigo blue and its absorption by chloroform.

Obermayer's Test

1. Add to about 5 cc. of urine in a test tube an equal volume of Obermayer's reagent and mix thoroughly.

Reagent	
Ferric chloride	2 grams
Hydrochloric acid (conc. sp. gr. 1.19)	1000 cc.

2. Heat until tube is warm.
3. Add 2 cc. chloroform and mix thoroughly by inverting, but avoid violent shaking.
4. Allow chloroform to settle.
5. If indican is present, the chloroform will be colored blue, depending upon the amount present. The indican in normal urine may give a very faint blue.
6. The urine of patients taking iodides may give a reddish-violet color which may obscure an indican reaction. By adding a few drops of concentrated sodium hyposulphite solution and shaking, the violet color will disappear, leaving the blue if indican be present. Occasionally, owing to slow oxidation, indigo red will form instead of indigo blue. This resembles the color given by iodides but does not disappear when treated with sodium hyposulphite.

Detection of Bile Pigments

Principles

The test given below depends upon the oxidation of bile pigments by acids with the formation of a series of colored derivatives like biliverdin (green) bilicyanine (blue) and choletelin (yellow). Bilirubin is perhaps the most important pigment.

Rosenbach's Modification of Gemlin's Test

1. Filter 100 cc. or more of a urine through a filter paper.
2. Remove the filter paper from the funnel and allow it to partially dry.
3. Touch the paper with a drop of old or yellow nitric acid.
4. If bile is present, a most marked spreading ring of rainbow colors with green on the outside will form.

Detection of Blood

Principles

The conditions in which blood occurs in urine may be classified under hematuria and hemoglobinuria. In the former, one is able to detect not only hemoglobin, but the unruptured corpuscles as well, whereas in the latter the hemoglobin alone is present. The detection of blood is usually detected by the color of the urine but the detection of traces requires microscopical and chemical examination. For the latter the usual tests for "occult blood" are required.

Benzidine Test

1. Prepare a saturated solution of benzidine base (Merck's) by dissolving a knife point full in 2 cc. of glacial acetic acid in a test tube. Warm if necessary.
2. Add an equal volume of 3% hydrogen peroxide.
3. Add 2 cc. of the urine and mix.
4. The appearance of a blue color indicates a positive reaction.

Orthotolidine Test

1. Solutions: a. 1% Sol of orthotolidine in methyl alcohol.
   b. Glacial Acetic acid 1 part - H_2O_2 two parts. (Sol. a, will keep several months; Sol b, one month).
2. Add two or three drops of sol. a to the sediment to be tested and then add two or three drops of sol. b.
3. Positive test is the appearance of a greenish-blue to a deep blue color.

URINARY SEDIMENTS

Turbidity of the urine is most often due either to bacterial contamination, amorphous urates (brick-dust sediment) or phosphates. In case turbidity is found, due to bacteria contaminating the urine, subsequent to its passing, it is best to call for another sample.

To preserve urinary sediments, formalin is best for casts and epithelial cells, while for general use one may employ a piece of camphor or 1 volume of saturated borax solution to 4 volumes of urine.

To take up the sediment for examination, insert a pipette to the bottom of the tube with the opposite opening closed by a finger; then holding the finger on the open end tightly, withdraw the pipette and deposit the sediment on a slide.

Unorganized Sediments: These are, as a rule, of little clinical interest, and give no reliable indication of abnormal production or excretion. The precipitation of any particular substance is dependent upon many physical as well as chemical factors, of which the reaction is very important. In amphoteric urines, one may encounter elements usually associated with either an acid or an alkaline reaction.

In a urine of ACID reaction, we may find the following:

1. Amorphous sodium or potassium acid-urates. Usually yellowish-red. Heat and alkali causes solution.
2. Uric acid. Yellowish-red crystals, usually of whetstone shape and in clusters and heaps. Soluable in alkalis but not by heat.
3. Calcium oxalate. Highly refractile octahedral crystals, or in dumbbell shapes. Often due to diet (asparagus, tomatoes, spinach, rhubarb, etc). Clumping of the crystals is suggestive when calculus is suspected.

4. Rare crystals such as cholesterol, cystin, tyrosin, leucin, xanthin, haematoidin, indigo, melanin, creatinine, hippuric acid, sodium biurate, etc.

In amphoteric urine, one may encounter dicalcium phosphate, or practically any other crystal.

In urine of ALKALINE reaction we may expect:

1. Triple phosphates. Usually coffin-lid crystals, or in fern-like forms. Easily soluble in acetic acid.
2. Tri-calcium phosphate, magnesium phosphate, and calcium carbonate. All are amorphous and easily soluble in acetie acid, the last with effervescence.
3. Ammonium biurate. Yellow, thorn-apple structures.
4. Calcium phosphate. Slender radiating crystals, or flat sheets.
5. Rarely, magnesium phosphate crystals.

The presence of ammonium biurate, particularly if with triple phosphates, denotes bacterial decomposition within the genito-urinary tract provided the urine is freshly passed. Pus cells derived from the site of inflammation should be present also. While certain bacteria might possible cause chemical changes without giving rise to inflammation, yet such a possibility is so rare as to be negligible. If amorphous phosphates are found, one should always consider exogenous sources such as vegetable diet, or special causes, as withdrawal of protein food.

Leukocytes: An occasional leukocyte may be found in the urine of healthy people. An abundance of leukocytes indicated inflammation of the genito-urinary tract. Some workers count the pus cells in urine by the same technic used for the leukocyte count of the blood. At times, the urine of women may contain an abundance of pus cells without pathological significance.

Erythrocytes: These may retain their biconcave form, be crenated, or only show as pale, possibly double-ringed bodies termed "shadows." They are however, usually quite uniform in size, a fact that will aid when one encounters, as he frequently does, other structures that closely resemble them, such as typical calcium oxalate crystals. Vegetable spores are often a source of confusion. It is well to always check a positive finding with a test for occult blood.

Epithelial Cells: It is almost impossible to state positively the origin in the genito-urinary tract of certain cells. A very trustworthy evidence, however, is finding of epithelial cells in cases of the so-called compound-granule cells (fatty degenerated renal epithelium). Sheets of more or less round or caudate epithelial cells are rather significant to the clinician. Vaginal epithelium resembles that secured by scraping the buccal mucosa. Bladder epithelium resembles vaginal but is of smaller size. Urethral is like that from the pelvis of the kidney, but smaller. Cells from the region of the prostate are very refractile, have a distinct necleus, and are oval rather than round.

Cylindruria: This means the presence of cylindroids or casts. It will be found that a 2/3 inch objective gives almost all the information required. Cylindroids are long structures resembling hyaline casts but showing tapering ends, irregularly in diameter, and longitudinal striations. They have the same significance as hyaline casts. Casts are cylindrical structures with rounded ends. One must bear in mind their fragility. It is said that prolonged centrifuging will disorganize them. They are of a light specific gravity and tend to occur in the upper portion of the sediment, either in centrifuge tube or on the slide. Todd says, "If the tubule be small and has its usual linging of epithelium the cast will be narrow; if it be large or entirely denuded of epithelium, the cast will be broad". Persistent presence is of graver significance than occasional occurance.

Hyaline casts are narrow and homogeneous. They follow almost any renal distrubance, and are not necessarily indicative of any permanent damage. Finely granular casts may have no greater significance than hyaline, and, as a matter of fact, the latter show a granular structure with dark-ground illimination. As the granules become coarser, it is generally considered that a more severe lesion is present. Fatty casts are especially associated with fatty degeneration. Epithelial casts are especially to be noted and reported, as the number present mean much to the doctor. Blood casts and pus casts mean a serious kidney condition and also should be noted and the number reported per low power field. Waxy casts are highly refractile and show fissuring of the margins. They may accompany a severe acute nephritis, and for that matter, should be reported. To summarize, always report the presence, kind, and size, and any cast noted in a urine, as they mean much to your doctor.

Starches and Fibers: In examining urinary sediments it is important to be familiar with the various textile fibers and starch grains which are so frequently present, the fibers coming from the clothing and the starch grains from dusting powder. Wool fiber fragments show bark or scale-like imbrications and are round. Cotton fibers are flattened and twisted, while linen ones show a striated, flattened fiber with frayed segments as of a cane stalk. Silk shows a glass-like tube with mashed in ends. Corn and rice are the most common of starch grains and their nature is immediately disclosed by their blue color when mounted in iodine. Scratches in the glass slide, or dust particles may be present in the specimen, but can usually be distinguished.

PREPARATION OF REAGENTS FOR URINALYSIS

Dilute acetic acid

Glacial acetic acid	5 cc.
Distilled water	100 cc.

Benedict's Qualitative Solution

Dissolve with the aid of heat.

Sodium citrate	173 grams
Sodium carbonate (anhydrous)	100 grams
Water . . . . about.	700 cc.

Filter solution if cloudy and then add slowly, with constant stirring:

Copper sulphate	.17.3 grams
Water	.100 cc.
Cool and dilute total volume to	1000 cc.

Haine's Qualitative Solution

Dissolve in about 300 cc. water: Sodium hydroxide - 32 grams. In another container dissolve in about 200 cc. water, with the aid of gentle heat: copper sulphate: 12 grams.

Mix the two solutions in a 1000 cc. graduated flask or cylinder and add 90 cc. glycerine. Dilute with water to liter mark and mix.

Ammonia Water

Regular U.S.P. Stronger ammonia.

Litmus

Supplied in both red and blue paper strips at all surgical supply or laboratory supply houses.

Sodium Nitroprusside

Sodium nitroprusside crystals	10 grams
Water	50 cc.

Mix the two and with aid of gentle heat dissolve the crystals.

Glacial acetic acid

Procurable at any drug store.

Robert's Reagent

Nitric Acid	1 part	167 cc.
Magnesium Sulphate Saturated Solution,	5 parts	833 cc.
		1000 cc.

Hydrogen Peroxide

Obtain at any drug store.

Tsuchiya's Reagent for Quantitative Albumin

Phosphotungstic acid	1.5 grams
Hydrochloric acid, concentrated	5 cc.
Ethyl alcohol, 95%	95. cc.

Keeps indefinitely.

Esbach's Solution

Picric acid (crystals)	10 grams
Citric acid (crystals)	20 grams
Distilled water	1000 cc.

Benedict's Quantitative Solution

Crystalized copper sulphate	18 grams
Anhydrous sodium carbonate (or double the weight of the crystalline salt)	100 grams
Sodium citrate	200 grams
Potassium sulphocyanate	125 grams
Potassium ferrocyanide (5% solution)	5 cc.
Distilled water, sufficient to make	1000 cc.

a. Dissolve the sodium carbonate, sodium citrate and potassium sulphocyanate in about 700 cc. water with the aid of gentle heat.
b. Filter.
c. Dissolve the copper sulphate in about 1000 cc. water.
d. Pour the copper solution into the other solution with constant stirring.
e. Add the ferrocyanide solution.
f. Cool and dilute to 1000 cc.

Lugol's Solution

Iodine, resublimed crystals	5 grams
Potassium iodide, crystals	10 grams
Distilled water	100 cc.

Benzidine Powder

Procurable at drug supply house. Specify Merck's Blue Label.

Gram's Iodine

Lugol's Solution..................................	1 part
Distilled Water...................................	14 parts

Obermayer's Reagent

Ferric Chloride	2 grams
Hydrochloric Acid.................................	1000 cc.

Buffer's Solution for Wright's Stain

Manobasic Potassium Phosphate....................	6.63 Gm.
Dibasic Sodium Phosphate	3.20 Gm.
H_2O..	1000 cc.

Gram's Iodine

Iodine, Resublimed crystals......................	1 Gm.
Potassium Iodide Crystals........................	2 Gms.
Distilled Water..................................	300 cc.

I. Sputum

A. Normal sputum may contain:
   1. Small rather dense mucoid masses - may be translucent or may be a gray, or may be pigmented.
   2. May have pus cells.
   3. May have endothelial cells.
   4. May have bacteria.
      a. Saprohytic bacteria
         (1) Staphylococcus.
         (2) Streptococcus.
         (3) Pneumococcus.
         (4) Micrococus Catarrhalis.
      b. Spirochetes
      c. Endamebae

B. Sputum should be brought up from the lungs. Mouth should be rinsed out in order that it be free of food particles and mouth debris. Be sure the specimen does not come from the nose or the naso-pharynx; must be placed in a clean recepti-cal and no disinfectants must be added until after examination.

C. The study of sputum should be divided in Physical Examination, Microscopic Examination and Chemical Examination.
   1. Physical Examination comprises:
      a. Quantity which may be in amount as being imperceptible to 1000 cc.
      b. Color which may be any shade from clear and transparent to colors and their shades through the yellows, greens, red and blacks.
      c. Consistency may comprise any degree in character from a thin water consistency to a very tenacious mass. May be serous, mucoid, purulent, a mucopurulant.
      d. May produce layer formation.
      e. Ditrich's plugs.
      f. Lung stones.
      g. Bronchial casts.
   2. Microscopical Examination consists:
      a. An unstained specimem should first be examined; this en-ables one to choose what particles of the sputum should be most productive of organism, or parts to be examined.
      b. In unstained sputum one should find elastic fibers, Cuschmann's spirals, Charcot-Leyden crystals, myelin globules, molds, pus corpuscles, mucus and granular detritis, also pigmented cells, heart failure cells.
      c. Stained sputum.
         (1) Several smears must be made of each specimen so that all stains can be utilized and all organisms, if pre-sent, will be stained.
         (2) Technique.
            (a) Proper specimen must be isolated; toothpicks or applicators should be used in preference to the wire loops. Those used must be disposed of pro-perly after their use.

(b) Material when placed on a slide must be allowed to dry.
(c) Fix in wood alcohol and 1% sol. corrosive sublimate for 2 min.
   Or pass through a flame with the film up several times, care being taken not to burn the film.
(d) Stain
   1-a. Carbol, Fuschin for T.B.
   2-a. Grams stain for other bacteria and their identification.
   3-a. Wright's stain for differentiation of white cells.

3. To stain T.B.
   a. Select the most purulent part, keeping away from the mucoid portion.
   b. May use Cabbet's Method or Zienl-Neelson's Method - this latter method is used most frequently.
      (1) Steam carbol fuschin on the smear for not less than 3 minutes; avoiding too much heat, so that it boils or allowing it to evaporate.
      (2) Wash until faint pink with either,
         (a) 5% HNO_3 Nitric Acid
         (b) Acid alcohol: 3 cc HcL; 97 cc Alcohol (70%)
      (3) Stain lightly with Loffler's blue or equal parts of alcohol and saturated solution of picric acid.

4. Tuberculosis Bacillus or Koch's Bacillus or the Acid Fast Bacillus, if present, will be seen as slender small rods in length, half the width of a R.B.C. They may be singly or in groups. T.B. Bacillus is to be differentiated from Smegma B., also edges of deeply stained material that has not decolorized and looks like bacilli, and an organism that comes off of Bermuda grass. Failure to find T.B. must call for several more examinations of the sputum and several more specimens of sputum. When definitely suspicious, sputum for T.B. is expected and not found by the usual methods. Concentration methods, cultural methods or animal innoculation methods are utilized.
   a. Loffler's method for concentration.
      (1) 10 cc. of sputum.
      (2) 10 cc. of 50% antiformin (equal parts of 15% solution of caustic soda and 20% solution of sodium hypochlorite).
      (3) Heat to boiling.
      (4) To each 10 cc. of fluid resulting, add 1.5 cc. of 1 vol. chloroform and 9 vol. alcohol.
      (5) Shake vigorously for several minutes.
      (6) Centrifuge for 15 minutes.
      (7) The Bacilli are in the layer over the chloroform which is at the bottom.
      (8) Transfer the sediment to slide.
      (9) Add a little original sputum.
      (10) Dry, fix and stain.
   b. Culture method is rarely done due to the difficulty in the growth of T.B. on artificial means much as media.

c. Animal innoculation of course is the court of last appeal and when negative, there are no T.B. present, however, there must be 10 to 150 bacilli present in the injected material, depending on the virulence of the organism to show positive pathology.

Other forms of Bacteria cells found in a stained specimen of sputum are: Staphylococci, Streptococci, Much's granules, Heart failure cells, Eosinophiles, Pneumococci, Friedlanders Bacilli, Influenza Bacilli, Micrococcus Catarrhalis, Higher Bacteria, Spirochetes, fungi and other leucocytes.

(a-1) An additional concentration method used at the Brooke General Hospital.

(1) Concentration method: Sodium Hydroxide: mix equal parts of sputum and a 3% sodium hydroxide solution, shake well, and incubate at 37° C. for fifteen to thirty minutes depending on the consistency of the specimen. Neutralize with normal hydrochloric acid, checking reaction with litmus. Centrifuge and prepare films from the sediment, fix, stain and examine as above.

EXAMINATION OF STOMACH CONTENTS

(1) Blood is present in the vomitus in a great variety of conditions. When found in the fluid removed after a test meal, it commonly points toward ulcer of carcinoma. Blood can be detected in nearly one half of the cases of gastric cancer. The presence of swallowed blood and blood from injury done by the stomach tube must be excluded.

Test for Blood in Stomach Contents: extract with ether to remove fat if this be present, which is usually not the case after a test meal.

To 10 cc. of the fat-free fluid, add 3 or 4 cc. of glacial acetic acid and shake the mixture thoroughly with about 5 cc. of ether. Let stand a short time, remove the ether, which forms a layer above the stomach fluid, and use half of it for the guaiac or benzidine test. Separation of the ether may be facilitated by adding a small amount of alcohol. In the case of a positive reaction the remainder of the ether extract may be examined spectroscopically after treating so as to develop the bands of hemochromogen.

When brown particles are present in the fluid the hemin test may be applied directly to them.

(2) Quantitative Tests.

(a) Total Acidity: the acid-reacting substances which contribute to the total acidity are free hydrochloric acid, combined hydrochloric acid, acid salts, mostly phosphates, and, in some pathologic conditions, the organic acids. The total acidity is normally about 50 to 100 degrees (see method below), or, when estimated as hydrochloric acid, about 0.2 to 0.3 per cent.

(b) Topfer's Method for Total Acidity: in an evaporating dish or small beaker take 10 cc. filtered stomach contents and add 3 or 4 drops of the indicator, a 1 per cent alcoholic solution of phenolphthalein. When the quantity of stomach fluid is small, 5 cc. may be used, but results are less accurate than with a larger amount. Add decinormal solution of sodium hydroxide drop by drop from a buret, until the fluid retains and there remains constant a faint, pink blush.

When this point is reached, all the acid has been neutralized. The end reaction will be sharper if the fluid be saturated with sodium chloride. A sheet of white paper beneath the beaker facilitates recognition of the color change.

In clinical work the amount of acidity is expressed by the number of cubic centimeters of the decinormal sodium hydroxide solution which would be required to neutralize 100 cc. of the gastric juice, each cubic centimeter representing one degree of acidity. Hence, multiply the number of cubic centimeters of decinormal solution required to neutralize the 10 cc. of stomach fluid by 10. This gives the number of degrees of acidity. The amount may be expressed in terms of hydrochloric acid, if one remembers that each degree is equivalent to 0.00365 Gm. of hydrochloric acid. Some one suggests that this is the number of days in the year, the last figure, 5, indicating the number of decimal places.

Example: Suppose that 7 cc. of decinormal solution were required to bring about the end reaction in 10 cc. gastric juice; then 7 x 10 = 70 degrees of acidity; and, expressed in terms of hydrochloric acid, 70 x 0.00365 = 0.255 Gm.

(c) Free Acidity.

Topfer's Method for Free Hydrochloric Acid: In a beaker take 10 cc. filtered stomach fluid and add 4 drops of the indicator, a 0.5 per cent alcoholic solution of dimethylamine-azobenzol. A red color instantly appears if free hydrochloric acid be present. Add decinormal sodium hydroxide solution, drop by drop from a buret, until the last trace of red just disappears, and a canary-yellow color takes its place. For accuracy it is better (Benedict) not to carry the titration quite to the canary-yellow stage, although the end point is then not so definite. Better still, when all the red has disappeared, read off the number of cubic centimeters of decinormal solution added, and calculate the degrees or percentage of free hydrochloric acid, as in Topfer's method for total acidity.

When it is impossible to obtain sufficient fluid for all the tests, it will be found convenient to estimate the free hydrochloric acid and total acidity in the same portion, and this is frequently adopted as a routine regardless of the amount of fluid available. After finding the free hydrochloric acid as just described, add 4 drops of phenolphthalein solution, and continue the titration. The total amount of decinormal solution used in both the titrations indicates the total acidity.

(3) Resume of Results.

(a) After the Ewald test breakfast, the amount of free hydrochloric acid varies normally between 25 and 50 degrees, or about 0.1 to 0.2 Gms. In disease it may go considerably higher or may be absent altogether. When the amount of free hydrochloric acid is normal, organic disease of the stomach probably does not exist.

(b) Increase of free hydrochloric acid above 50 degrees (hyperchlorhydria) generally indicates a neurosis, but also occurs in most cases of gastric ulcer and beginning of chronic gastritis. It has been found in normal persons.

(c) Decrease of free hydrochloric acid below 25 degrees (hypochlorhydria) occurs in some neuroses, chronic gastritis, early carcinoma, pellagra, anemias, and most conditions associated with general systemic depression. Marked variation in the amount at successive examinations strongly suggests a neurosis. Too low values are often obtained at the first examination, the patient's dread of the introduction of the tube probably inhibiting secretion.

(d) Absence of free hydrochloric acid (achlorhydria) occurs in most cases of gastric cancer and far-advanced chronic gastritis, in many cases of pellagra, and sometimes in hysteria and pulmonary tuberculosis. Achlorhydria is a constant and important symptom of pernicious anemia even during remissions. It sometimes appears long before any anemia is recognizable.

(e) The presence of free hydrochloric acid presupposes a normal amount of combined hydrochloric acid, hence the combined need not be estimated when the free acid has been found. When, however, free hydrochloric acid is absent, it is important to know whether any acid is secreted, and an estimation of the combined acid then becomes of great value. The normal average after an Ewald breakfast is about 10 to 15 degrees, the quantity depending upon the amount of protein in the test meal. Somewhat higher figures are obtained after a Riegel test meal. Of greater significance than the amount of combined acid is the acid deficit, described later.

(f) For the determination of combined hydrochloric acid, a 1% aqueous solution of sodium alizarin sulfonate is used as an indicator. Titration is done the same as described under free acidity above. The end point is at the time of the appearance of purple color. Is also calculated as above the amount being determined by subtracting the results of this titration from the amount of predetermined total acidity.

ANTIGENS, ANTIBODIES, AND IMMUNITY

In the worst epidemics in the history of the world, there have always been a few survivors, those who recovered from the disease, and those who did not even contract the disease. These people must have had some natural protection which helped them recover or prevented the disease entirely. This is called natural immunity. When some disease organisms invade the body, the body develops a protective condition which prevents that disease organism from producing disease again. This is called acquired immunity. The substance which stimulates the production of this protection is called an antigen. Antigen is chemical in nature, seems to be protein, and may come from either bacteria or cells. The protecting substance which is formed to react against the antigen is called the antibody. There are several types of antigens, antibodies, and reactions produced by them as shown in the table below.

ANTIGEN	ANTIBODY	REACTION
Toxin	Antitoxin	Neutralization (makes non-poisonous)
Agglutinogen (bacteria or cells)	Agglutinin	Agglutination (causes clumping)
Precipitinogen (soluble proteins)	Precipitin	Precipitation (separates from solution)
Lysinogen (bacteria or cells)	Lysin	Lysis (dissolving)
Bacteriocidinogen (bacteria)	Bacteriocidin	Kills bacteria
Opsoninogen (bacteria or cells)	Opsonin	Increases phagocytosis (ingestion of substance by special cells)
Complement fixing Antigen (bacterial, cellular, or protein)	Amboceptor	Complement fixation

The above reactions serve as a basis for a number of tests done in the laboratory. The reactions are specific for both antigen and antibody. Therefore if a known antigen is added to an unknown antibody and the specific reaction is produced, the specific antibody must have been present. If no reaction is produced, the antibody was not present. Also if an unknown antigen is added to a known antibody and the specific reaction is produced, the specific antigen must be present. If the reaction is not produced, the specific antigen is not present. Special procedures and techniques are necessary to accomplish these tests.

SEROLOGICAL TESTS FOR SYPHILIS

TESTS PRESCRIBED

For uniformity and for efficiency of supply, Circular Letter #10 dated February 13, 1941, from the Surgeon General's Office makes several provisions for tests for syphilis to be used in the Army of the United States.

The qualitative Kahn flocculation test is to be used for all routine tests.

The Wassermann test with anti-sheep hemolytic system will be used for spinal fluid and for confirmatory tests on new doubtful, and positive serums of patients with no other positive clinical symptoms.

Method of reporting

Results of tests will be reported as "positive", "doubtful", or "negative."

The system "+", "±", and "-" will not be used.

Records

The first specimen of blood of a patient submitted to any Army laboratory will be accompanied with Form 97MD properly executed. It will be kept at the laboratory as a permanent record of serological tests done for that particular patient.

Each specimen of blood or serum submitted for test for syphilis will be accompanied by Form 55L-3MD properly executed in duplicate. The original is returned to the sender with recorded results. The duplicate is kept by the laboratory as a temporary record and the report entered in the proper space on Form 97 MD for permanent record.

Each specimen of spinal fluid will be accompanied by Form 55L-4MD properly executed in duplicate. Results of the test will be reported to the sender on the original and the duplicate will be kept in the laboratory with results recorded in proper place on Form 97 MD for permanent record.

All the above forms are first made out or initiated by the surgeon making the request for the test.

Every laboratory doing any test for syphilis on a patient will have a form 97MD for that patient.

THE STANDARD KAHN TEST

REAGENTS

1. Physiological salt solution

Sodium chloride, CP. 8.50 Grams
Distilled water 1000.00 Ml.
Filter if necessary before using. The solution need not be sterile but must be chemically pure.

2. Standard Kahn Antigen

Medical Department Supply Catalog, Class 1, order thus: (Item #) 17030-ANTIGEN FOR KAHN TEST, 50 CC:(unit)bottle. Secured on quarterly requisition or on emergency requisition from the Army Medical Center, Washington, D.C.

It is prepared by the Army Medical School, Army Medical Center, Washington, D.C. It is an alcoholic extract of powdered beef heart that has had the fat removed by ether extraction, and has had cholesterol added to increase its sensitivity. The solution has been standardized to a degree of specificity and sensitivity required for the standard Kahn tests and will maintain this specificity and sensitivity many years if the following precautions are taken:

a. Keep stored in the dark. Mailing container satisfactory.
b. Store at room temperature only.
c. Keep tightly stoppered at all times.
d. Stopper must be adequately covered with high grade tin foil. Rubber and cork contain alcohol soluble substances which affect specificity.
e. Only dry, chemically clean glass vessels shall be used to store the antigen.
f. Only dry, chemically clean pipettes shall be used to measure the antigen during preparation for the test.

To prepare antigen suspension for the Kahn tests proceed as follows:

a. Place required amount of antigen in one tube (never less than 1 ml.)

b. Place required amount of saline in second tube (quantity indicated by titre stated on label of antigen bottle, as:
Titre 1 1.4 means that for each 1 ml. of antigen use 1.4 ml. of saline).

c. Pour saline into antigen quickly, then pour back and forth 12 times.

d. Allow to age for ten minutes before use, but use before thirty minutes for best results.

3. Unknown Serum

a. Eight to ten ml. of blood are collected by venipuncture with the usual aseptic technique from the patient to be tested, then place blood in a clean dry tube. Water causes hemolysis.

b. Allow blood to stand one hour at room temperature for clot to form. Do not agitate because hemolysis may result.

c. Free the clot from the walls of the tube with a clean dry applicator. Use separate, clean applicator for each tube.

d. Centrifuge at 2000 RPM for ten minutes or place in a refrigerator and allow clot to retract, and preserve until ready to use.

e. Decant or pipette serum into a clean dry tube. Recentrifuge if cells are present in serum.

f. Place tube of serum in a water bath at 56°C for one-half hour to inactivate.

g. Test serum as soon after inactivation as possible. Serums not inactivated for five to twenty-four hours should be inactivated again for ten minutes. If over twenty-four hours, inactivate for fifteen minutes.

Serums will vary in color because some will have a small amount of hemolysis causing a red color, some will have chyle causing a cloudy serum, and some will have an increased amount of bile causing an amber or brownish color. There must be no contamination with water or other substance.

4. Known Positive Serum

Serum previously tested and known to be "four plus", - i.e., strongly positive. It must be inactivated before use which is done in the same manner as the unknown serum.

Blood may be withdrawn by venipuncture from a person recently tested and known to be strongly positive. The serum is prepared in the same manner as the unknown serum.

5. Known Negative Serum

Serum previously tested and known to be negative. It must be inactivated before use in the test.

Blood may be withdrawn by venipuncture from a person recently

tested and known to be negative. The serum is prepared in the same manner as the unknown serum.

APPARATUS

1. Blood tubes, 2 for each control serum and for each blood tested (one for whole blood and one for serum).

2. Kahn tubes, 9 for controls and 3 for each unknown tested.

3. Kahn rack, one for each 30 tubes.

4. Vials or tubes for mixing antigen, 2, 10 to 25 ml.

5. Pipettes, 1 ml. graduated to 0.01 ml., 3 for controls and 1 for each unknown serum.

6. Pipettes, 0.2 or 0.25 ml. graduated to 0.0125 ml., 1 to use for antigen.

7. Pipettes, 5 or 10 ml. graduated to 0.1 ml.

8. Centrifuge and balance.

9. Water bath adjusted to 56°C.

STANDARD KAHN PROCEDURE - on next page.

STANDARD KAHN PROCEDURE (ALL MATERIALS HAVING BEEN PREPARED)

	Antigen	SERUM		Saline		RESULTS
UNKNOWN			SHAKE FOR THREE MINUTES		SHAKE ENOUGH TO MIX	
Each Unknown Serum						See below
Front tube	0.05 ml.	0.15 ml.		1.0 ml.		
Middle "	0.025 "	" "		0.5 "		
Rear "	0.0125 "	" "		0.5 "		
CONTROLS:						
Known positive		pos. serum				Marked flocculation, clear fluid
Front tube	0.05 ml.	0.15 ml.		1.0 ml.		
Middle "	0.025 "	" "		0.5 "		
Rear "	0.0125 "	" "		0.5 "		
Known negative		Neg. serum				No flocculation, cloudy fluid
Front tube	0.05 ml.	0.15 ml.		1.0 ml.		
Middle "	0.025 "	" "		0.5 "		
Rear "	0.0125 "	" "		0.5 "		
Saline control		Saline				No flocculation, cloudy fluid
Front tube	0.05 ml.	0.15 ml.		1.0 ml.		
Middle "	0.025 "	" "		0.5 "		
Rear "	0.0125 "	" "		0.5 "		

RESULTS: Check controls first. If unsatisfactory, repeat entire test.

Negative – Solution remains cloudy, no flocculation present in any of the three tubes.

Positive – Varying degrees of flocculation and loss of cloudiness of fluid. Read each tube separately, report average, as front tube 3 plus

25% flocculation 1 plus	Doubtful		middle "	3 plus
50% " 2 "			rear "	1 "
75% " 3 "			Total	7
100% " 4 "	Positive		Average	2 plus
			Report	"doubtful"

QUANTITATIVE KAHN TEST PROCEDURE

To be done only on serum showing three or four plus reaction with Kahn test.

1. Blood collected by venipuncture is allowed to clot, then is centrifuged to separate the serum. Serum must be entirely free of cells.

2. Inactive serum in water bath at 56°C for 30 minutes.

3. Mix Kahn Antigen
   a. Place 1 ml. antigen in one vial or tube.
   b. Place required amount of saline in second vial or tube. (quantity indicated by titre on label of antigen bottle)
   c. Pour saline into antigen quickly, then pour back and forth twelve times. Allow it to age ten minutes.

4. Prepare serum dilutions as follows while antigen is aging:

Tube #	Saline		Serum or serum dilution	Dilution Ratio
1 ____	0.6 ml	+	0.4 ml. serum	1:2.5
2 ____	0.5 "	+	0.5 " of dilution from tube #1	1 : 5
3 ____	" "	+	" " " " " " #2	1 : 10
4 ____	" "	+	" " " " " " #3	1 : 20
5 ____	" "	+	" " " " " " #4	1 : 40
6 ____	" "	+	" " " " " " #5	1 : 80

If necessary the dilutions may be carried higher.

5. A one-tube Kahn test is then done on each of the above dilutions as follows: place six tubes in a rack and proceed as shown. Start with tube # 6.

Tube #	Antigen	Serum		Saline	
6 -	0.025 ml.	0.15 ml of 1 : 80 dil.	Shake 3 minutes	0.5 ml.	Shake to mix & read
5 -	" "	" " " 1 : 40 "		" "	
4 -	" "	" " " 1 : 20 "		" "	
3 -	" "	" " " 1 : 10 "		" "	
2 -	" "	" " " 1 : 5 "		" "	
1 -	" "	" " " 1 :2.5 "		" "	

6. Results:
   a. The highest dilution which shows a three plus or four plus reaction indicates the quantitative dilution.
   b. Reporting, two methods used.
      (1) By units - Quantitative dilution x 4 = no. of units. Example - 1 : 20 dilution is highest dilution showing a three plus reaction, so; 20 x 4 = 80 units, the quantitative reading.
      (2) By reporting the reaction of each tube starting with tube # 1, as:

++++, ++++, ++++, +++, +, -

TITRATION OF KAHN ANTIGEN

Kahn antigen is very stable if properly taken care of. It occasionally becomes necessary, however, to check the titre because of possible improper care.

By determining the titre one determines the amount of physiological saline to add to each ml. of antigen when preparing it for test.

To determine this amount various amounts of saline are added to 1 ml. amounts of antigen. A precipitate is formed in each instance. When a specified amount of saline is added to each, the precipitate redissolves in some tubes and does not in others. The antigen-saline mixture having the least amount of saline in which the precipitate redissolves in further addition of saline indicates the quantity of saline to add to each 1 ml. of antigen, in other words the titre.

To accomplish the titration, set up 25 tubes as shown below.

PROBLEM: To check ANTIGEN TITRE LABELED 1 + 1.4

Antigen-Saline mixes to be tried: Tube	Pour saline (tube a) into antigen (tube b). Pour back and forth twelve times. Then pipette each in- to respective 3 tubes in amounts as shown.	Test each of the Antigen-saline mixtures as follows: Tube	Antigen	Saline	Shake vigorously for 3 minutes and add saline as follows:	Saline	Shake enough to mix
1a Saline 1.2 ml.		Front	0.05 ml.	0.15 ml.		1.0 ml	
1b Antigen 1.0 "		Middle	0.025 "	" "		0.5 "	
		Rear	0.0125"	" "		0.5 "	
2a Saline 1.3 ml.		Front	0.05 "	" "		1.0 "	
2b Antigen 1.0 "		Middle	0.025 "	" "		0.5 "	
		Rear	0.0125"	" "		0.5 "	
3a Saline 1.4 ml.		Front	0.05 "	" "		1.0 "	
3b Antigen 1.0 "		Middle	0.025 "	" "		0.5 "	
		Rear	0.0125"	" "		0.5 "	
4a Saline 1.5 ml.		Front	0.05 "	" "		1.0 "	
4b Antigen 1.0 "		Middle	0.025 "	" "		0.5 "	
		Rear	0.0125"	" "		0.5 "	
5a Saline 1.6 ml.		Front	0.05 "	" "		1.0 "	
5b Antigen 1.0 "		Middle	0.025 "	" "		0.5 "	
		Rear	0.0125"	" "		0.5 "	

The saline-antigen mixture with the least amount of saline in which the precipitate completely redissolves is the mixture or titre to use for the test.

When checking antigen of lower titre, use smaller amounts of saline for each ml. of antigen. For example, if antigen is labeled

1 + 1.1, make mixes 1 + 0.9; 1 + 1.0; 1 + 1.1; 1 + 1.2; 1 + 1.3. If higher titre, use larger amounts of saline.

SPINAL FLUID KAHN PROCEDURE

1. Centrifuge spinal fluid until free of cells.

2. Place in a centrifuge tube the following:

   a. Clear spinal fluid . . . . 1.5 ml.
   b. Saturated Ammonium sulfate solution. . 1.5 " .

3. Place centrifuge tube in water bath 56°C. for 15 minutes. A white cloudiness or precipitate indicates the presence of globulin.

4. Centrifuge 3000 RPM for 10 minutes to pack globulin in bottom of tube.

5. Decant and discard supernatant fluid. Drain remaining supernatant fluid by inverting the centrifuge tube on a clean towel for several minutes.

6. Dissolve precipitate by adding 0.15 ml. of 0.85% saline, then shaking.

7. Do a Kahn test on this globulin solution as follows:

   a. Mix Kahn Antigen.

      (1) Place 1 ml. antigen in one vial or tube.
      (2) Place required amount of saline into second vial or tube. (Quantity indicated by titre on label of antigen bottle.)
      (3) Pour saline into antigen quickly, then pour back and forth twelve times. Allow it to age 10 minutes.

   b. Pipette into bottom of a Kahn tube.

      (1) Antigen. 0.01 ml.
      (2) Globulin solution. 0.15 "
          Shake vigorously for 3 minutes.
      (3) Add Saline. 0.5 ml.
          Shake to mix.

8. Results:

   Positive - Flocculation with loss or clearing of cloudy appearance.

   Negative - No flocculation, no change, no loss of cloudiness.

THE COMPLEMENT FIXATION TEST

History - In 1888 Nuttall demonstrated bacteriolysis, the process of dissolving bacteria. He mixed a measured amount of blood serum or defibrinated blood with a measured amount of certain bacteria. He incubated the mixture for several hours at 37°C. He then poured several plates with this mixture in the media. He also poured several plates with the bacteria alone without the serum. After allowing time for growth, he demonstrated that the plates containing the serum had many less colonies, some of them being nearly sterile. This showed that there was something in the serum that inhibited the growth of or killed many bacteria.

Nuttall also demonstrated that if the serum was heated to 55 or 56° C. or was allowed to stand or age, it would no longer kill or inhibit the growth of the bacteria.

In 1894 Pfeiffer demonstrated bacteriolysis to be specific, that is, if a specific bacterium is injected into an animal, that animal develops a bacteriolysin, the substance producing bacteriolysis. This bacteriolysin will dissolve that particular bacteria but no others. This he demonstrated in the following manner.

Into the peritoneal cavity of a guinea pig which had recovered from cholera was injected a suspension of cholera spirilla. At regular intervals after the injection, exudate was withdrawn from the peritoneal cavity. The first specimens withdrawn showed swelling of the spirilla, later specimens showed distortion in shape, granulation in appearance, and finally complete disappearance.

Other bacteria injected into the peritoneal cavity of a guinea pig recovered from cholera were not thus affected thus showing the action to be specific.

Pfeiffer also observed that guinea pigs that had recovered from cholera would tolerate large doses of cholera spirilla injected into their peritoneal cavities, whereas other guinea pigs died from small doses.

He also observed that if the peritoneal cavity of a normal pig was injected with a mixture of cholera spirilla and the blood serum from a guinea pig recovered from cholera, the normal pig escaped infection. This proved that a normal guinea pig could be protected from the disease, that the blood carried the same bacteriolysins that were present in the peritoneal cavity as shown above.

Pfeiffer also showed that the immunized serum, serum from guinea pigs recovered from the disease, as used in the above experiments, was used just as effectively if heated as if not heated when injected in the peritoneal cavity of the guinea pigs. But if used in the test tube, the heated serum did not dissolve the spirilla.

Bordet showed that if a small amount of fresh normal serum was added to the heated immune serum, bacteriolysis or dissolving of the bacteria would also take place in the test tube.

Bordet's experiments showed that unheated immune serum depends on three substances - all necessary for bacteriolysis:

1. A specific bacterium used to produce the immunity.
2. Blood serum containing the protective substance.
3. A substance present in all normal serum.

In 1898 Bordet reported hemolysis, the power to dissolve or lake red blood cells. He showed that if a rabbit be injected with a man's red blood cells, a hemolysin was produced that would cause laking of the red blood cells in the presence of the serum of this rabbit. If the serum was heated to 56°C. for one-half hour, and the red blood corpuscles of man added, there would be no laking. If normal serum was added to this mixture, a hemolysis occurred. This reaction could be carried out for any two species of animals, i.e., rabbit and man, sheep and rabbit, etc.

It is apparent therefore that three substances are necessary here also: (1) Erythrocytes used to sensitize serum, (2) sensitized or immune serum, and (3) normal serum.

The above experiments have given rise to the following terms and definitions:

ANTIGEN - Any substance which when injected into suitable animals, will result in the formation of specific antibodies.

AMBOCEPTOR - The specific antibody produced by the injection of an antigen. Amboceptors may be normally present in certain blood sera but can be greatly increased by the injection of the suitable antigen. Amboceptors are thermostable, that is, retaining their activity after heating at 56°C. for one-half hour.

A BACTERIOLYSIN is a bacteriolytic amboceptor.
A HEMOLYSIN is a hemolytic amboceptor. The first dissolves bacteria and the second dissolves red blood cells.

COMPLEMENT - The substance present in all normal fresh sera, which when added to a mixture containing antigen, and amboceptor, results in the production of bacteriolysis or hemolysis. Complement is rendered inactive by heating for one-half hour at 56° and is therefore said to be thermolabile. It also loses strength rapidly on standing at room temperature.

BACTERIOLYTIC SYSTEM - The combination of antigen (bacterium), amboceptor (immune serum), and complement (normal serum).

HEMOLYTIC SYSTEM - The combination of antigen (erythrocytes), amboceptor (immune serum), and complement (normal serum).

In the above two systems all three substances must be present or no reaction takes place, i.e., antigen, amboceptor, and complement.

Since a bacterial antigen produces a specific antibody or amboceptor, it was reasoned that a test for the presence of this antibody or amboceptor in the blood serum should be possible. With this test it should be possible to tell whether a patient has or has had an infection from a certain bacterium because of the production of

these specific antibodies or amboceptors which would be in the blood.

Therefore if a patient's serum with complement present dissolves the cholera spirilla, the patient must have cholera or must have had it. If it dissolves the spiracheta pallida, the causative organism of syphilis, the patient must have syphilis.

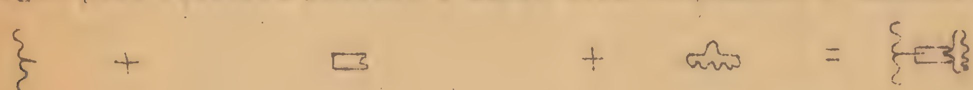
Because bacteriolysis is difficult to visualize, practically, the test is carried further.

The amount of complement necessary to produce complete bacteriolysis can be determined for any measured amount of patient's serum and antigen. If the patient's serum has the specific amboceptor, the complement will be used or fixed.

To determine if any or all of the complement is fixed, a hemolytic system is added, that is, red blood cells and rabbit serum sensitized or immunized to those cells. In order for hemolysis to take place, complement is necessary. So if this hemolytic system is added to the bacteriolytic system and there is some complement left, some hemolysis will take place. If no complement is left, no hemolysis can take place. Hemolysis is easily visualized and readily shows whether any or all the complement is used or fixed.

The reaction may be visualized as follows:

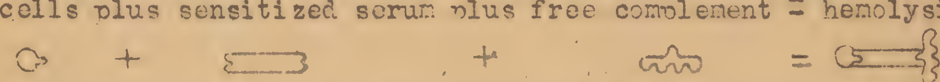
Antigen plus Positive Patient's serum plus complement = bacteriolysis



and no free complement.

Antigen plus neg. Pt's serum plus complement = no bacteriolysis

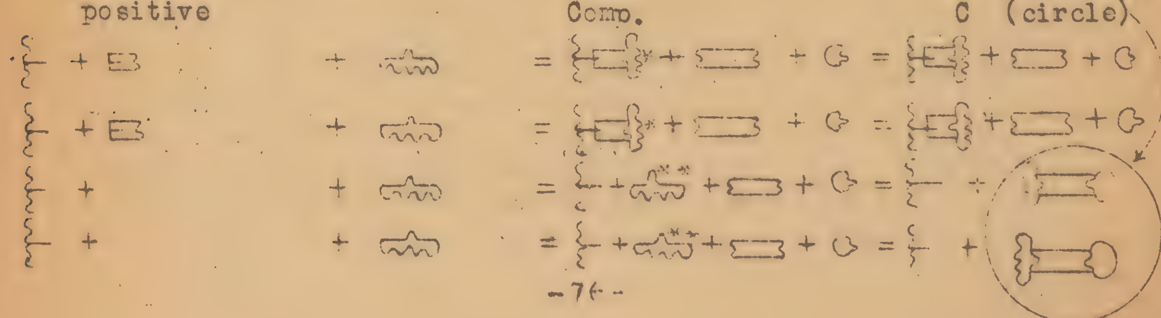
When hemolytic system is added to the above the following occur:
Red blood cells plus sensitized serum plus free complement = hemolysis



R.B.C. plus sensitized serum plus no complement = no hemolysis

Variation in positivity of serum may be demonstrated thus:

Antigen + Pt's Serum +	Complement =	Fixed* & +	Amboceptor + R =	Partial
partly		Free**	B	hemolysis
positive		Comp.	C	(circle)



THE TWO-TUBE KOLMER TEST
(Sheep System)

A. Glassware and Apparatus.

Pipettes: 0.2 cc, graduated to 0.01 cc
1.0 cc, graduated to 0.01 cc
10.0 cc, graduated to 0.1 cc

Test tubes, 100 x 12 mm., heavy wall, without lip.
Test tube racks, carrying two rows of ten tubes each.
Centrifuge and centrifuge tubes
Water baths: Inactivating, set at 56 deg. C.
Incubating, set at 37 deg. C.
Refrigerator, running at 6 to 8 deg. C.

All glassware should be chemically clean and should be used dry or rinsed out with normal saline solution just before using. Never use any glassware containing the slightest degree of water.

B. Reagents.

1. Patient's serum. Blood is collected from the patient by venipuncture and allowed to clot. The serum should be separated from the clot and centrifuged until all cells are thrown to the bottom of the tube. The serum is poured or pipetted from the top and placed into a clean tube. The serum must be entirely free of cells. Before the test is run, all sera are inactivated in 56 degree water bath for 30 minutes.

2. Salt solution. This is an isotonic solution of sodium chloride. Add 0.85 grams of chemically pure sodium chloride (Merck's Blue Label) to 100 cc of distilled water.

3. Sheep Cell Suspension (indicator Antigen). Collect the blood by bleeding the sheep from the external jugular vein into 1 to 3 percent sodium citrate solution, or the blood may be received into a flask containing a handful of sterile glass beads and shaken well to defibrinate it. Either method prevents clotting. The former method is preferable. Filter a small amount of blood through cotton into graduated centrifuge tube, allowing twice as much blood as will be required for the test to be run. Add 2 or 3 volumes of salt solution. Centrifuge at 1500 R.P.M. for ten minutes. Decant and add saline. Repeat this washing 5 times. On the last washing centrifuge at the 1500 R.P.M. for exactly 15 minutes. Do not vary the time or speed, so as to insure the same per cent suspension when the cells are finally diluted for use in the test. Read the volume of the cells in the centrifuge tube, carefully remove the supernatant fluid, and prepare a 2 % suspension by washing the cells into a flask with 49 volumes of salt solution. Always shake well before using to secure an even suspension, as the cells rapidly settle to the bottom of the flask on standing.

The sheep cells can be preserved for a period up to three weeks by placing one volume of cells in four volumes of saline and adding for each 100 cc, 1 1/4 cc of formalin (40% formaldehyde). When the cells are to be used they are washed three or four times with saline as above.

4. Complement. The serum of guinea pigs is used. Animals are selected which have not been recently bled. The complementary strength will be more uniformly good if the pooled serum of several guinea pigs is used.

The animal is placed on its back with an ether cone over its nose. When the animal has relaxed (care: too much ether causes death), blood is withdrawn from the heart with a 10 cc syringe and 18 or 20 gauge short bevel, sharp needle, the needle being carefully placed through the chest wall into the ventricle. When 8 or 10 cc have been secured, withdraw the needle and place the animal aside on its back to recover. Remove the needle from the syringe and gently run the blood into a centrifuge tube. Do not stir or agitate because this causes hemolysis. Set aside for an hour at room temperature to allow clot to form. Free the clot from the walls of the tube with a clean sterile applicator. If it is to be kept over night, place in a refrigerator. When it is to be used, centrifuge at 1500 R.P.M. for 15 minutes. Decant or pipette off the serum. If it contains cells, recentrifuge. The serum should show no hemolysis and contain no cells. It must be used when fresh because it loses its complementary strength. It can be kept over night if the clot is not separated from it. The serum is diluted with saline in the amount determined by titration.

Lyophilized complement is pooled guinea pig serum which has been frozen and dried under vacuum in ampules, each of which contains a specified amount of serum. In this form, it retains its complementary strength for many months. The dried serum is regenerated for use by adding enough distilled water to bring it to its original volume. This serum is then diluted and titrated in the same manner as is fresh serum. This lyophilized serum is obtained from the Army Medical Center on quarterly or emergency requisition, is considered as deteriorating, and is listed as follows:

Item No.		Unit	Price
17034	COMPLEMENT FIXATION TEST, LYOPHILIZED, GUINEA PIG, 5 CC	bottle	$3.00

It must be stored in refrigerator.

5. Amboceptor. Serum of rabbits sensitized to sheep cells. It is diluted to 50% with glycerine to preserve it. Keep in a refrigerator. It need not be inactivated. It is prepared at, and is secured from the Army Medical Center, Washington, D. C., on a quarterly or emergency requisition. It is considered as a deteriorating item and is listed as follows:

Item No.	Unit	Price
17000 - AMBOCEPTOR, ANTISHEEP, HEMOLYSIN, 5 CC	Bottle	$6.00

6. Antigen. It is a cholesterolized and lecithinized alcoholic extract of ether extracted powdered beef heart. The same precautions should be taken with this antigen as with the Kahn antigen. It is kept at room temperature. It should be titrated for antigenic activity about every three weeks. It is prepared at and secured from the Army Medical Center, Washington, D. C. on a quarterly or emergency requisition. It is considered as a deteriorating item and is listed as follows:

Item No.	Unit	Price
17020 - ANTIGEN, SYPHILIS COMPLEMENT FIXATION TESTS, 5CC..............................	Bottle	$3.60

7. Known Positive Serum. Serum previously tested and known to be strongly positive. It must be free of cells and inactivated.

8. Known Negative Serum. Serum previously tested and known to be negative. It must be free of cells and inactivated.

C. TITRATIONS

1. Titration of Amboceptor

a. Prepare a dilution of 1:100 amboceptor as follows:

Glycerolized amboceptor (50%)	2 cc
Salt solution	94 cc
Phenol (5% in salt solution)	4 cc

This is to be kept in the refrigerator as a stock solution and is good for several weeks.

b. Dilute this stock solution for the titration as follows:

Stock amboceptor (1:100)	0.5 cc
Salt solution	4.5 cc

This will be 1:1000 in strength.

c. In a series of 10 tubes, prepare higher dilutions as follows:

# 1.	Amboceptor	1:1000,	0.5 cc plus no saline						
# 2.	"	"	0.5 cc	plus	0.5	cc	saline	(1:2000)
# 3.	"	"	"	"	"	1.0	"	"	(1:3000)
# 4.	"	"	"	"	"	1.5	"	"	(1:4000)
# 5.	"	"	"	"	"	2.0	"	"	(1:5000)
# 6.	"	1:3000	"	"	"	0.5	"	"	(1:6000)
# 7.	"	1:4000	"	"	"	0.5	"	"	(1:8000)
# 8.	"	1:5000	"	"	"	0.5	"	"	(1:10000)
# 9.	"	1:6000	"	"	"	0.5	"	"	(1:12000)
#10.	"	1:8000	"	"	"	0.5	"	"	(1:16000)

Mix the contents of each tube thoroughly.

d. Prepare a 1:30 dilution of the complement by diluting 0.2 cc of the regenerated complement or fresh guinea pig serum with 5.8 cc of salt solution.

e. Prepare a 2% suspension of sheep cells in salt solution.

f. In a series of 10 tubes set up the amboceptor titration as shown in the following table:

Tube	Amboceptor 0.5 cc	Complement 1:30	Saline	Sheep Cells
1.	1:1000	0.3 cc	1.7 cc	0.5 cc
2.	1:2000	to all	to all	to all
3.	1:3000	tubes	tubes	tubes.
4.	1:4000			
5.	1:5000			
6.	1:6000			
7.	1;8000			
8.	1:10000			
9.	1:12000			
10.	1:16000			

Mix the contents of each tube thoroughly.

g. Incubate in the water bath at 37 deg. C. for 1 hour.

h. Read the amboceptor unit. The unit is the highest dilution of amboceptor that gives complete hemolysis.

Two units of amboceptor are used in the complement and antigen titrations and in the final test. Example: If the unit equals 0.5 cc of the 1:6000 dilution, then two units will equal 0.5 cc of the 1:3000 dilution. Dilute just enough of the stock amboceptor for the titrations and the number of tests to be run.

2. <u>Titration of Complement.</u>

a. Prepare a 1:30 dilution of the complement. (See paragraph d under amboceptor titration)

b. Dilute the antigen as indicated by the dilution factor on the antigen bottle, by placing the required amount of salt solution in a small flask and adding the antigen drop by drop, shaking the flask continually until the antigen has all been added. Prepare enough for the complement titration and for the final test.

c. In a series of 10 tubes, set up the titration as follows:

Tube	Complement (1:30) cc	Antigen Dose cc	Salt Solution cc		Amboceptor 2 Units cc	Sheep Cells 2% cc	
1.	0.1	0.5	1.4	Water	0.5	0.5	Water
2.	0.15	0.5	1.4	Bath	0.5	0.5	Bath
3.	0.2	0.5	1.3	37°C	0.5	0.5	37°C
4.	0.25	0.5	1.3	for	0.5	0.5	for
5.	0.3	0.5	1.2	one	0.5	0.5	one
6.	0.35	0.5	1.2	hour	0.5	0.5	hour
7.	0.4	0.5	1.1		0.5	0.5	
8.	0.45	0.5	1.1		0.5	0.5	
9.	0.5	0.5	1.0		0.5	0.5	
10.	None	None	2.5		None	0.5	

The smallest amount of complement just giving sparkling hemolysis is the exact unit. The next higher tube is the full unit, which contains 0.05 cc more complement. In the antigen titration and in the final test, two full units are used and are so diluted as to be contained in 1.0 cc as in the following example:

Exact unit	0.3 cc
Full unit	0.35 cc
Dose (2 full units)	0.7 cc

To calculate the dilution to employ so that 1.0 cc will contain the dose of 2 full units, divide 30 by the dose (0.7).

This equals 43, therefore 1.0 cc of a 1:43 dilution will contain the required 2 full units.

D. Titration of Antigen

1. Prepare a 1:80 dilution of antigen by adding 0.1 cc, drop by drop, with continual shaking, to 7.9 cc of salt solution in a large test tube or a small flask.

2. Higher dilutions are then prepared as follows:

4 cc of 1:80	+ 4 cc of salt solution	1:160 dilution
4 cc of 1:160	+ 4 cc of salt solution	1:320 dilution
4 cc of 1:320	+ 4 cc of salt solution	1:640 dilution
4 cc of 1:640	+ 4 cc of salt solution	1:1280 dilution
4 cc of 1:1280	+ 4 cc of salt solution	1:2560 dilution

3. Arrange 5 rows of test tubes with 6 tubes in each row (30 tubes)

4. In the first tube of each row place 0.5 cc dilution 1:80.
In the second tube of each row place 0.5 cc dilution 1:160.
In the third tube of each row place 0.5 cc dilution 1:320.
In the fourth tube of each row place 0.5 cc dilution 1:640.
In the fifth tube of each row place 0.5 cc dilution 1:1280
In the sixth tube of each row place 0.5 cc dilution 1:2560

5. Heat 3 cc of moderately to strongly positive syphilitic serum in a water bath at 56 deg. C. for 15 to 20 minutes and prepare 5 dilutions in large test tubes as follows:

Tube	Serum, cc	Saline, cc	Resulting Dilution	CC of Serum in 0.5 cc of Dilution
1	1.0	4.0	1.5	0.1
2	0.5	4.5	1:10	0.05
3	0.5	9.5	1:20	0.025
4	2.0 (1:20)	2.0	1:40	0.0125
5	1.0 (1:20)	4.0	1:100	0.005

6. Add 0.5 of 1:5 dilution to each of the 6 tubes of the 1st row.
Add 0.5 of 1:10 dilution to each of the 6 tubes of the second row.
Add 0.5 of 1:20 dilution to each of the 6 tubes of the third row.
Add 0.5 of 1:40 dilution to each of the 6 tubes of the fourth row.
Add 0.5 of 1:100 dilution to each of the 6 tubes of the fifth row.

7. Add 1.0 cc of complement dilution carrying 2 full units to all 30 tubes.

8. In a separate rack, set up a serum control carrying 0.5 cc of 1:5 serum and 1.0 cc of complement (2 full units); also a hemolytic system control carrying 1.0cc of salt solution and 1.0 cc of complement (2 full units).

9. Shake the tubes gently and place in the refrigerator at 6 to 8 degrees C. for 15 to 18 hours, followed by water bath at 37 deg. C. for 10 minutes.

10. Add 0.5 cc of amboceptor (2 units) and 0.5 cc of a 2% suspension of sheep cells to all 30 tubes and to the control tubes.

11. Mix thoroughly and place in water bath at 37 deg. C. for one hour and make readings. The serum and hemolytic controls should show complete hemolysis.

12. Chart the results as per the following example observed with a strongly positive serum:

Serum (in 0.5 cc)	Antigen dilutions (in 0.5 cc dose) 1:80	1:160	1:320	1:640	1:1280	1:2560
0.005	-	-	++	-	-	-
0.0125	-	+	++++	++++	++	+
0.025	+	++++	++++	++++	++++	+
0.05	+++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	+++

The dose of antigen to employ in the final test is the largest amount giving a ++++ reaction with the smallest amount of serum. If three dilutions of antigen give ++++ reactions with the smallest amount of serum, the dose is midway between the highest and lowest.

E. Procedure for the Test

Having ascertained the exact amounts of the reagents to be used by the above methods, set up the two-tube Kolmer test on the various blood sera for diagnosis as indicated in the table on the following page.

PROCEDURE FOR TWO-TUBE KOLMER TEST ON BLOOD.

	Tube No.	Serum cc.	Antigen	Allow to stand 10 minutes at room temperature	cc Complement (2 units)	Incubate in refrigerator at 6-8°C for 15-18 hours	Place in water bath at 37°C for 10-15 minutes.	Hemolytic System: cc Amboceptor (2 units)	Hemolytic System: Sheep Cells 2% susp.	Incubate in water bath at 37°C for 1 hour	Results
Unknown Serum "Test"Tube	1	0.2	0.5		1.0			0.5	0.5		++++=no hemolysis) Report as +++=25% " Positive ++ =50% ") + =75% ") Doubtful - =100% ") Negative
"Serum Control"	2	0.2	NONE (0.5 cc saline)		1.0			0.5	0.5		"Serum Control" tube should show complete hemolysis. If not, report anticomplementary serum.
Known Positive Control	1	0.2	0.5		1.0			0.5	0.5		No Hemolysis
	2	0.2	(0.5 cc saline)		1.0			0.5	0.5		Complete Hemolysis. If not serum is anticomplementary
Known Negative Control	1	0.2	0.5		1.0			0.5	0.5		Complete hemolysis
	2	0.2	NONE (0.5 cc saline)		1.0			0.5	0.5		Complete hemolysis. If not serum is anticomplementary
Antigen Control	1	NONE (0.5 cc saline)	0.5		1.0			0.5	0.5		Complete hemolysis
Amboceptor Control	2	NONE (1.0cc saline)	NONE		1.0			0.5	0.5		Complete hemolysis
Sheep Cell Control	3	NONE (2.5 cc saline)	NONE		NONE			NONE	0.5		No hemolysis

Spinal Fluids.

These are usually tested without any preliminary preparation as they do not contain enough complement to require inactivation by heating at 56°C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged.

The following table shows the set-up for complement fixation tests on spinal fluids for syphilis:

Tube No.	Spinal Fluid cc	Antigen cc.		Complement (2 units)		Amboceptor (2 units) cc.	Sheep cells (2%)	
1	0.5	0.5	Wait ten minutes room temperature.	1.0	Incubate in refrigerator at 6 to 8°C. for 15 to 18 hrs., then 10 to 15 minutes in water bath at 37° C.	0.5	0.5	Incubate in water bath at 37° C. for one hour.
2	0.5	None		1.0		0.5	0.5	

Tube number 2 is the control tube and should show complete hemolysis. The antigen, amboceptor and sheep cell control should be run with each lot, the same as for blood serum.

A control, consisting of a known positive and a known negative fluid should also accompany each lot of fluids tested.

EXAMINATION OF CEREBROSPINAL FLUID

Collection - Collect cerebrospinal fluid in two chemically clean sterile tubes. Label 1 and 2. The first specimen may contain blood from the needle puncture. No. 2 is reserved for the cell count. Examination should be completed as quickly as possible after collection. If it is necessary to send the fluid to a distant laboratory for the Wassermann and colloidal gold tests, the cell count, study of smears, and the simpler chemical tests should be done in the local laboratory and reported on a slip accompanying the specimen.

I. Appearance

Normally crystal clear, colorless - like clean water.

Color:
1. Colorless - Normal
2. Yellow - Altered hemoglobin (old blood), jaundice.
3. Red - Hemoglobin or blood.

Transparency:
1. Crystal clear - Normal
2. Slight Haziness - Blood or pus cells.
3. Turbid - Blood or pus cells.

Coagulum:
1. No coagulation - Normal.
2. Pellicles - Forms after standing some hours due to meningeal inflammation.
3. "Cobweb" or "Pine Tree" Coagulum - Tuberculous meningitis.

II. Cytology - Cell counts must be made with a fresh specimen because cells degenerate rapidly.

A. Material -
1. Spinal Fluid
2. Staining solution
   - Methyl or Gentian violet........0.2 gms.
   - Glacial acetic acid............10.0 cc..
   - Distilled water................90.0 cc.

   Mix well, filter until the solution is clear.
3. Wright's stain 3 parts + methyl alcohol 1 part; buffer.
4. Leucocyte pipette.
5. Fuchs-Rosenthal counting chamber or ordinary Hemocytometer.
6. Microscope.
7. Glass slides.
8. Methyl alcohol.

B. Procedure for Counting

1. Shake spinal fluid to get uniform suspension of cells.
2. Fill pipette to 0.1 mark with stain.
3. Fill with spinal fluid to 1.1.
4. Shake well.
5. Fill counting chamber and allow to stand for 3 minutes.
6. Count cells in entire ruled area.
   Fuchs-Rosenthal - 16 squares
   Hemocytometer - 9 squares
7. Calculation
   (a) Fuchs-Rosenthal - chamber 4 x 4 mm. x 0.2 mm. equals 3.2 cu. mm. The 0.2 being nearly equal to the proportion of dilution by stain is disregarded hence:

   $$\frac{\text{Total count}}{3} = \text{Number of WBC per cu. mm.}$$

   (b) Hemocytometer - chamber is 3 mm x 3 mm x 0.1 mm. equals 0.9 cu. mm.

   $$\frac{\text{Total count} \times 10}{9} = \text{Number of WBC per cu. mm.}$$

   Again calculation compensates for small error caused by dilution.
8. Results - Normal is 0 to 8 cells per cu. mm.

C. Procedure for Differential Count
1. Centrifuge spinal fluid for 5 minutes.
2. Prepare thin smears of sediment on glass slides.
3. Dry in air quickly.
4. Stain with Wright-methyl alcohol stain.
5. Make differential count, polymorphonuclear and lymphocytes.

Results: Normal - Lymphocytes and few endothelial cells.
Abnormal - Polymorphonuclears.

PREPARATION OF COLLOIDAL GOLD SOLUTION

METHOD OF PREPARATION AT EIGHTH SERVICE COMMAND LABORATORY

I. Solutions.

1. Fifteen grains of gold chloride (ampoule by Merck) are made up to 100 cc. with double distilled water in a volumetric flask.

2. Five grams of sodium citrate (Merck, U.S.P.) are weighed out accurately on an analytical balance and made up to 500 cc. with double distilled water in a volumetric flask.

II. Procedure.

Nine hundred fifty cubic centimeters of double distilled water are measured out with a one liter graduate into a three-liter Florence flask. Ten cubic centimeters of gold chloride, measured with a 10 cc. volumetric pipette, are added to the double distilled water. This solution is slowly brought up to 94°C; and at that point of temperature, 50 cc. of the 1% citrate measured in a 50 cc. volumetric flask are added all at once to the gold chloride water solution. Immedicately the flame is turned to full force. As soon as the liquid has definite large boiling bubbles, two and one-half minutes are timed with a time clock. As soon as the two and one-half minutes are over, turn the flame out and set the colloidal solution aside to cool.

NOTE: Scrupulously clean glassware must be used throughout the above procedure. We make a practice of using glassware which is not used for anything but colloidal gold. All glassware should be washed two or three times with double distilled water before using. The second distillation of water should be done with a full glass still, using tinfoil to cover the bottles after distillation. A large gas burner is required for boiling.

Standardization of Colloidal Gold.

A. Apparatus
   1. 12 chemically clean test tubes
   2. Test tube rack for tubes
   3. Pipettes: 5 ml. graduated to 0.1 ml.
                1 " " " 0.01 ".

B. Reagents
   1. Colloidal gold solution
   2. Saline, 0.4% to be kept in chemically clean bottle
      Sodium chloride, CP 1 gram
      Double distilled water 250 ml.
   3. Synthetic globulin furnished by Army Medical School.

C. Procedure

Prepare a 1:60 dilution of the synthetic globulin by mixing 0.1 cc of globulin with 5.9 cc of .4% saline. The diluted globulin is good for 1 day's use only and must be prepared fresh for each day.

Place 10 chemically clean test tubes in a rack and place in each tube solutions as indicated:

Tube No.	Saline 0.4%, mix each tube well before proceeding.		
1	0.9 ml.	+ 0.1 ml.	Synthetic globulin, mix thoroughly.
2	0.5 "	+ 0.5 "	mixture from tube #1, mix
3	0.5 "	+ 0.5 "	" " " #2, "
4	0.5 "	+ 0.5 "	" " " #3, "
5	0.5 "	+ 0.5 "	" " " #4, "
6	0.5 "	+ 0.5 "	" " " #5, "
7	0.5 "	+ 0.5 "	" " " #6, "
8	0.5 "	+ 0.5 "	" " " #7, "
9	0.5 "	+ 0.5 "	" " " #8, "
10	0.5 "	+ 0.5 "	" " " #9, "

Remove 0.5 ml. from tube #10 and discard.

Control Tube:
   Saline 0.4% 1.0 ml.
Then place in each tube including the control tubes -
   Colloidal Gold Solution 2.5 ml.

Mix thoroughly by rotating tubes. Allow to stand at room temperature 18 to 24 hours.

D. Results

The readings depend upon the presence or absence of color change. For convenience, colors are given numbers as follows:

#0 - No color change
#1 - Slight violet tinge
#2 - Bluish red
#3 - Blue
#4 - Light transparent pink or blue almost decolorized
#5 - Complete decolorization.

Control - With 4% saline, no color change, or "0".

A gold of standard potency should exhibit the following:
Zone 1 - Curve, 5,554,310,000

A record of the reaction of each gold prepared and used should be kept. More than a slight deviation from the above curve warrants discarding of the gold and another prepared.

Colloidal gold should be allowed to stand 24 hours before testing. Should be stored in sterile bottles using either ground glass stoppers or tin foil protected stoppers.

Colloidal Gold Test on Spinal Fluid

Same procedure as that of standardization by synthetic globulin, except 0.1 cc spinal fluid undiluted is placed in tube # 1.

Reporting - The color of each tube is reported by number. The following are examples of normal and abnormal reports:

	Tubes #1 to #10 inclusive
Normal	0,000,000,000 2,210,000,000
"First Zone" or "Paretic"	5,555,542,100 5,554,310,000
"Mid Zone", "Luetic" or "Tabetic"	0,123,221,100 2,443,100,000 3,455,542,100
"End Zone" or "Meningitic" (Showing shift to right)	0,001,223,310 0,002,455,555

NOTES

NOTES

LABORATORY TECHNICIANS MANUAL

PART II

INDEX

Page 97 missing

Glass Handling

1. Cutting of glass tubing and test tubes.
2. Bending of glass tubing.
3. Making capillary pipettes from glass tubing.
4. Preparing an ampule out of a test tube.
5. Blowing of bulbs in glass tubing or test tubes.
6. Sealing joints in glass tubing.

In the average laboratory there is some call for turning glass tubing or test tubes into a form for some special purpose, and the faculty for doing this may be readily acquired with practice.

Equipment: only soft glass can be so handled with the ordinary laboratory equipment; the hard glasses, such as "Pyrex" having too high a melting point to be handled without special high temperature blowpipes. The standard 6, 8 and 10 mm. thick wall tubing and the standard test tube will suffice for the average requirements. While some simple manipulation may be done on a bunsen burner, it is preferable to have a blast lamp, both to provide higher temperature and to have smaller controllable heat point. Most blast lamps are designed for use with artificial gas; difficulty may be encountered in using natural gas, of higher B.T.U., with these burners and where natural gas is used special modification of the burners may be required. Satisfactory gasoline blast burners are also on the market. A small triangular file provides the means of cutting the glass.

Cutting of Glass Tubing

Place the glass tubing on a table, hold it firmly and nick it in one spot by firmly drawing across it the edge of a triangular file. (It is rarely necessary to extend this file nick around the tubing.) Then holding the tubing in both hands with both thumbs opposite the nick a quick snap will complete a clean cut break of the tubing. If the break is not clean cut it indicates the need of deeper nick or of a modified manipulation in effecting the snap, a technic to be acquired with practice. If one end of the tubing is too short to so handle, the snap may be effected by holding the long end rigidly in one hand and hitting the small end by the file held in the other hand. After the break any sharp points, from failure to attain a satisfactory clean break, may be filed down. The surface of the break is finally smoothed by melting it in a hot flame; at this time, by overmelting it, the bore at the top can be reduced to any desired size.

Of course the hot glass should not be laid down on the table top; an asbestos board, the tip of a wire basket or a metal ring stand may be so used.

Cutting a Test Tube

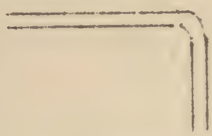
Make the file nick as for glass tubing but make it deeper and preferably encircle the tube. A thin tube may be broken at this point by a bi-manual snap. Thick tubes require additional aids to complete the break: at one point make the file nick especially deep, then touch the tube firmly at this point with the red hot file tip - a fracture should then result; if the fracture is not complete it may be traced around the tube by keeping the red hot tip just ahead of the fracture line, on a cold test tube. The trim up is the same as for tubing.

Bending of Glass Tubing

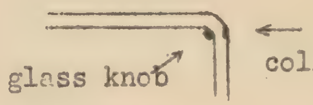
Holding both ends place the tubing in a hot flame so that at least an inch gets hot. Rotate tube while it is heating to make the heat even on all sides. When the glass is red hot and soft remove from flame and bend to the desired form keeping it in that position until it has hardened. If a broad bend is desired, as in making a "U" bend, several inches should be so heated. If only a slight bend is to be made, an inch of glass will suffice. At first you will have a tendency to overheat the glass and draw the two ends apart, distorting the shape and caliber. Also if you underheat, or put forced pressure on the bending effort, an undesirable collapse of the tubing at the bend will occur. A satisfactory bend retains the same caliber throughout the tubing.

If, in working with thin tubing, the collapse at the bend cannot be prevented there is a device available for preventing it: having sealed one end of the tubing before melting it, the mouth is applied to the other end while effecting the bend, and enough air pressure is made into the tubing to return the collapsed tubing to its proper form - a procedure similar to the blowing of glass bulbs described below.

Satisfactory Bend



Unsatisfactory Bend



Making Capillary Pipettes from Glass tubing

Glass tubing is heated, rotated, in flame to softness, removed from flame and the two ends drawn apart and held in place until hardened. The size of the resultant capillary tubing will depend on the degree of heat, the rapidity of drawing out and the extent of the drawing out. The tendency is to make the tube too small by too rapid separation of a narrow length of heated tubing. There is frequent use in bacteriology for such a pipette in this form.

It is desirable to keep on hand 8 inch lengths of glass tubing, with cotton plugs at both ends, the whole sterilized by dry heat for use in making into pipettes as desired for special uses. Such pipettes may be given a narrowing of lumen about 3/4 inch from the end, to prevent the cotton plug from passing deeper into the pipette.

Preparing an ampoule out of a test tube

Proceed with a test tube just as above drawing, the two ends only about two inches apart resulting in a neck of about 4 mm. diameter. The tube may be cut at this point converting the lower end into an ampoule to be later sealed, or the test tube may be left intact to be later sealed at the constriction. Excessive heat may make tube difficult to hold; this may be avoided by placing a large perforated cork over each end and holding and rotating these.

Blowing bulb in Glass Tubing

One end of the glass tubing is sealed and sufficient length allowed for holding one end in hand, the other end in mouth while blowing. Holding the tube over the flame with both hands rotating it for even heat, it is given a red heat to the melting point, then passed to the mouth to blow up the bulb to the required size. If the bulb is to be of considerable size some concentration of glass must be attained before the final blow; this is done by gently approximating the two ends while the middle is soft, giving an occasional slight blow to prevent collapse of melted glass. Trouble encountered will consist of eccentric bulbs, due to uneven heating, or to thin paper shell bulbs due to overblowing without glass concentration. A test tube may be similarly handled. A terminal bulb may be made at end of glass tubing by a one hand manipulation and blowing.

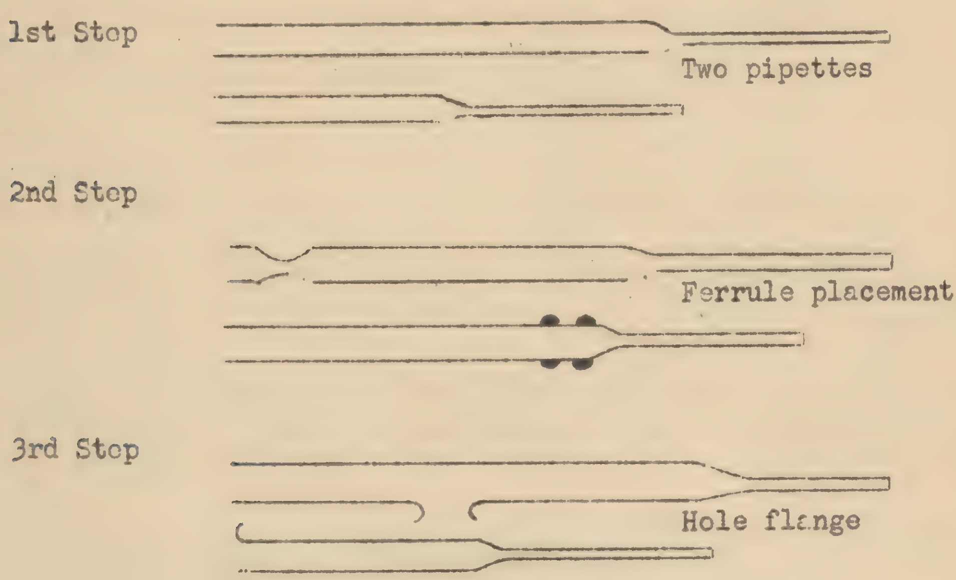
Union of Glass Tubing

If two straight pieces of glass are to be spliced, one piece is first given a seal at one end, the two ends to be spliced are given a slight flange by gently rotating within the heated end, the sharp end of a triangular file. The two flanged ends should be of same size, shape, and same grade of glass; they are heated to a red heat and then approximated to complete sealing on all sides, there results a union which is oversize and too thick; this union may be trimmed off to even size with the tubing by heating over the flame, while rotating evenly, blowing slightly into the tubing occasionally as tendency to collapse occurs, and slightly separating the two ends and blowing slightly if over thick glass is noted at the union.

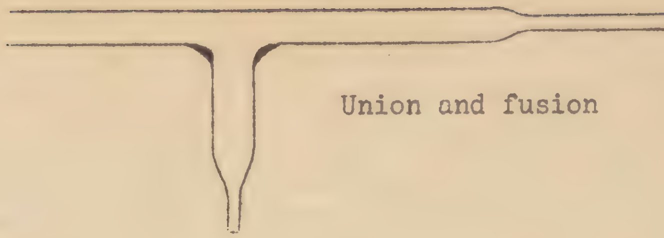
By similar procedures glass tubing of different size or glass tubing and test tubes may be united. A hard glass united with a soft glass would tend to fracture at the union. A union having thick spots or knobs will tend to fracture on change of temperature.

<u>Making joints in glass tubing</u>

The making of Y of glass tubing, that is, a three way tube, combines the process of blowing a bulb and union of two glass tubes. We begin with glass tubing of any desired size. Two lengths are prepared, each sealed at one end, such as being drawn to capillary length. One of these is given a flange at open end, the flange molded into an oval shape by the action of end of file on the molten tube. The other piece of glass is given a hole in its center to receive the flange: the tubing is held in a small hot flame, without rotation, to heat only one small spot of the tubing; when it is melted a lopsided bulb is blown to an extreme thinness even to rupture; then the bulb is crushed away with the file leaving the hole surrounded by the ragged bulb fragments; heat is applied to the edges of this hole to make it evenly heated and the same size as the flange on the other piece of tubing. When both the flange and the hole borders are at molten heat the two are brought gently together, being sure of union at all points but avoiding collapse. The union is then smoothed off by alternate heat and blowing as in the simple union except that rotation cannot be effected and each side has to be separately fused. A tendency to attain lumps of glass must be prevented and these smoothed out if they occur, otherwise this will become a weak point in the joint. The three ends of the union are then cut to the desired length. Inasmuch as these joints are generally used for holding rubber tubing a desirable refinement would consist of giving them, prior to making the joint, a double ferrule of glass at the end of each future arm of joint.

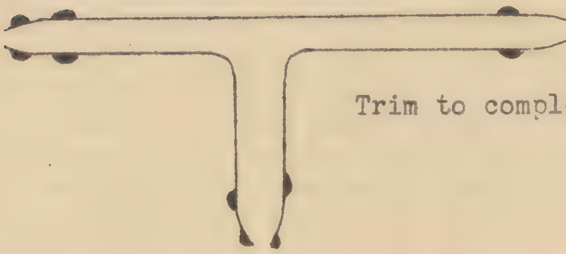


4th Step



Union and fusion

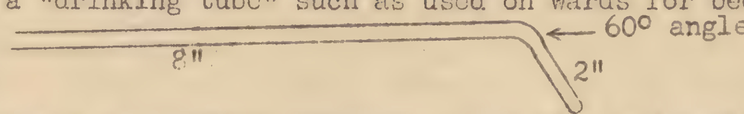
5th Step



Trim to completion

Exercise in Glass Work:

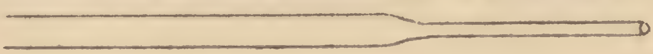
1. Prepare a "drinking tube" such as used on wards for bed patients.



(try using it yourself to determine best size and angle.)

2. Prepare a "U" tube such as used in laboratory bottles.

3. Prepare a capillary pipette such as will be used for specimen taking.



4. Prepare a glass bulb about the size of a walnut: Round, smooth, even.

5. Prepare a union of a piece of 6 to a piece of 10 mm. tubing.

6. Prepare a "Y" or "T" joint.

7. As each of the above is completed to your satisfaction, deliver it to the instructor on a slip bearing your name and a note that you have personally done all parts of the making of the piece.

Equipment of this work:

Blast lamp & foot bellows
Glass tubing: 6 & 10 mm.
File
Asbestos board
Wire basket
Receptacle for waste glass

FEEDING, CARE AND BREEDING OF LABORATORY ANIMALS

Laboratory Animals: (1) rabbit, (2) Guinea pig, (3) mouse, (4) albino rat and (5) monkey.

Reception Quarantine: All animals received from an outside source, should be isolated for 10 days to 3 weeks in previously disinfected quarters, and found to be free from disease before mixing with regular stock.

Housing: Animal quarters should be kept clean, dry and completely free from vermin. The optimum temperature for most animals is 65° to 70°F. with adequate ventilation. The standard large ($10\frac{1}{2}$") and small (8") animal jars are suitable for mice and rats; the large jar also can be used for a small guinea pig. The standard galvanized iron animal cage (14" x 14" x 16") will hold one rabbit or several guinea pigs. For use in breeding rabbits or guinea pigs, larger cages or pens, preferably with outside runways, should be built. The bottom of the jar or tray in cage should contain an absorbent bed material, such as wood shavings; hay or straw may be used in large breeding cages. Clean quarters and renew bedding twice per week.

Rabbits:

(1) The diet recommended consists of commercial "Rabbit Pellets" supplemented once or twice per week with feeding of green stuff, such as carrots, lettuce or celery tops. A diet consisting of equal parts of oats, wheat and barley, plus 10% of logume, soybean or linseed meal is suitable. Alfalfa or timothy hay will serve both for food and bedding. Always keep plenty of water and a small piece of rock salt in the cage.

(2) Diseases:

a. "Coccidiosis", an intense and fatal onteritis, is the most serious disease. Observe new rabbits for this several days before adding to stock.

b. "Ear Mange" is caused by a mite; can be cured by local application of a parasiticide.

c. "Snuffles" is a cold-like disease caused by a filterable virus. Isolate infected rabbits until 3 weeks after recovery.

(3) Breeding: Keep one male (buck) for each 8 to 10 females (does). Females are ready for mating at age of 10 months and may be bred every 3 months thereafter (4 litters per year). Keep record of date bred; gestation period 31 days; 2 or 3 days before expected arrival of litter place small breeding box and ample supply of bedding in cage. Wean young after 8 weeks and separate sexes.

Guinea Pigs:

(1) Feeding: Same as for rabbits, except they must have supplementary feeding of green stuffs to supply Vitamin "C", at least twice per week.

(2) Diseases:

a. Salmonella infections, chiefly Salmonella typhimurium and S.enteritidis, are most dangerous of common diseases. Best method of control: Kill all potentially infected animals, sterilize room and cages and obtain new stock.

b. Vitamin "C" deficiency is caused by lack of sufficient green stuffs in diet. Characterized by coarse hair and mangy appearance. It is transmissible to young through mother. Treatment: - improved diet.

c. Balantidium coli type of enteritis.

(3) Breeding: Use colony breeding with 4 or 5 females in cage with one male; duration of pregnancy - 63 days. Wean young and separate sexes when 4 or 5 weeks old.

Mice: Several different strains used, such as, white mice, Swiss mice (also white) and C 57 strain (black).

(1) Feeding: Commercial dog or fox chow checkers furnish an ample, balanced diet for growth and breeding; occasionally add piece of carrot or other greenstuff. Must have supply of fresh clean water in cage at all times. Mice will do well on simpler diets, such as, (a) the mixed grain diet listed above for rabbits, or (b) dry bread with water or skimmed milk, with addition of cod liver oil once per week.

(2) Diseases:

a. Salmonella infections (mouse typhoid), caused by same organisms as for guinea pigs, are common and very dangerous. To control: - destroy all infected stock, sterilize room and cages and obtain fresh stock.

(3) Breeding: Colony breeding, with 4 or 5 females to one male; gestation period 21 days; when well advanced pregnancy is observed, place female in individual jar. After 21 days, isolate young and return mother to breeding jar. Feed young same as adults, but addition of evaporated milk to diet hastens growth.

Albino Rats:

(1) Feeding: Same as for mice.

(2) Diseases: If cages are kept clean and ample diet provided, rats are very resistant to disease.

(3) Breeding: Young females are ready for breeding when 4 months old. Use colony method of breeding with 4 females and one male in cage; duration of pregnancy 22 days; not necessary to remove pregnant female from breeding cage. Wean young and separate sexes when 21 days old.

Monkeys:

(1) Feeding: Monkeys will do very well on "dog-chow checkers" plus canned tomatoes, with occasional feeding of fruits and nuts (oranges, apples, bananas, peanuts, sunflower seeds, etc.).

(2) Diseases:

a. Pneumonia, usually fatal.

b. Miliary tuberculosis.

(3) Breeding: In captivity in small laboratories is not practical.

1. Bacteria are stained by the <u>basic</u> aniline dyes. The <u>acid</u> aniline dyes, including eosin and acid fuchsin are not suitable for bacterial staining. The basic aniline dyes mentioned below, and others, if needed, may be conveniently kept as stock solutions of the powder in alcohol to saturation, from which are prepared various simple and compound staining solutions.
2. <u>Stock</u> solutions of dyes consist of these dyes in saturated alcoholic solution. They are prepared by placing the measured amount for solution, or slightly more, into 95% ethyl alcohol at room temperature and, after shaking for complete solution, filtering through paper to remove surplus dye and debris. Label "________, stock solution".

Dye	Solubility in 100 cc. of 95% alcohol
Crystal violet - - - - - - - - - - - - - - - -	13.87 gms.
Fuchsin (<u>basic</u>)- - - - - - - - - - - - - - - -	8.16 gms.
Methylene blue - - - - - - - - - - - - - - - -	1.48 gms.
Safranin -	3.41 gms.

Example: To prepare stock solution of safranin: Place slightly in excess of 3.41 gms. of the dye in 100 cc. of 95% alcohol, shake to solution over period of 2 or 3 days, filter through paper, label.

3. Simple stain solutions:
   General formula: Stock dye solution- 10 cc.
   Distilled water - - 90 cc.
   Uses: Simple stains or as elements of compound stains..
   Application: Apply stain to a fixed slide for 2-5 minutes, wash with water, blot dry.
4. Loeffler's Alkaline Methylene Blue:
   Formula: Potassium hydroxide 10% sol. .07 cc.
   Distilled water - - - - - -- - 70 cc. mix and add
   Methylene blue, stock solution 30 cc.
   Preparation: The KOH is first added to H_2O to make 1:10,000 dilution, then dye added.
   Uses: General bacterial stain.
5. Carbol-fuchsin, dilute:
   Formula: Carbol-fuchsin (formula elsewhere) 10. cc.
   Distilled water - - - - - - - - 90. cc.
   Uses: General bacterial stain.
6. Bismark Brown (not kept as a stock alcoholic solution).
   Bismark brown powder - - - - - - - - - - .5 gm.
   Boiling water - - - - -- - - - -- - - - - -- 100.0 cc. Cool & filter
7. Gram's Method: This is the most important of all bacteriological stains. It includes the application in turn, to a fixed slide, of a violet stain, Gram's iodine, a decolorizing agent and a contrast counterstain.
   Reagents: (a) <u>Primary stain</u>: Crystal violet - ammonium oxalate solution
   Crystal violet, stock solution - - - - - - - 5. cc.
   Alcohol, 95% - - - - - - - - - - - -- -- - - - - 5. cc, mix and add
   Ammonium oxalate, 1% aqueous solution - - - 40. cc.
   (b) Gram's iodine:
   Iodine - 1. gm.
   Potassium iodide - - - - - - - - - - - - - - - - 2. gms.
   Distilled water - - - - - - - - - - - 240.) or distilled
   Sodium bicarbonate, 5% aqueous solution-60.) water 300 cc.

(c) Decolorizer: 95% ethyl alcohol, or acetone, or alcohol-acetone (50-50).

(d) Counterstain: Any simple contrast stain: Safranin, bismark brown, or dilute carbol-fuchsin.

Technique of Gram-staining:

(1) Prepare thin even slide spreads, air dry, pass through flame for fixation.

(2) Crystal violet stain is applied for 1 minute, then excess stain is poured off.

(3) Gram's iodine is then applied for 1 minute. Wash in Water.

(4) Decolorizer is applied in several washes until no further traces of the stain can be washed out of the preparation (1/2 to 2 minutes). Wash in water.

(5) Apply counterstain (e.g. safranin) for ½ minute. Wash in water. Blot and air dry.

Results:

Gram-positive organisms are stained violet.
Gram-negative organisms are stained pink (brown or red)
Gram-ambophile organisms give a variable result.

General rules of Gram behavior of organisms:

(1.) Cocci are Gram-positive except gonococcus, meningococcus, and catarrhalis group.

(2.) Bacilli are Gram-negative except the diphtheria, the acid-fast group and most spore bearers.

(3.) Spirilla and spirochetes are Gram-negative.

(4.) Older cultures of Gram-positive organisms tend to become Gram-ambophile or negative.

8. Neisser's Method for Polar Body Staining.

Formulae:

(1) Polar body stain ("Neisser #1")
Methylene blue - stock solution - - - - - - - - - - - - - 10 cc.
Acetic acid, 5% solution, freshly prepared - - - - - 50 cc

(2) Counterstain: ("Neisser "2")
Bismark brown - (formula elsewhere)
(or use the safranin as prepared for Gram's counterstain)

Technic of Stain:

1. Prepare even, thin spreads on slides, air dry and fix by heat.
2. Apply polar body stain for 1 to 3 minutes, wash.
3. Apply counterstain for 1 minute. Wash and dry.

Result:

Polar bodies will be stained blue; bacillary bodies take the counterstain.

Uses:

Differential stain of the diphtheria bacillus, the plague bacillus and others having metachromatic granules.

9. Acid-fast Stain Method (Ziehl-Neelsen's carbol-fuchsin).

Formulae: Includes a primary stain, a decolorizer and a counterstain.

1. Carbol-fuchsin:	Basic fuchsin, stock solution------	10. cc.
	Phenol, 5% solution----------------	90. cc.
2. Acid alcohol:	Acid, hydrochloric------------------	3. cc.
	Alcohol, ethyl, 95%----------------	97. cc.
3. Counterstain:	Loeffler's Methylene blue.	

Technic:

1. Prepare spreads of suspect material on slides, air dry and fix by heat.
2. Apply carbol-fuchsin and heat gently until steam appears over the surface. Allow to steam for 5 minutes. Wash in water.
3. Decolorize with acid alcohol by renewal washer to a faint pink. Wash in water.
4. Counterstain with Methylene blue for ½ minute, wash in water, blot dry.

Results:
Acid-fast organisms are stained red.
Non-acid-fast organisms are stained blue.

Application: The detection of tubercle bacilli, leprosy bacilli and a few other acid-fast organisms.

10. Hiss' Method for Capsules.

Formulae:

1. Staining solution: Crystal violet, stock solution-----10. cc.
   Distilled water--------------------90. cc.
   (Gram's crystal violet or Ziehl-Neelsen's carbol-fuchsin may be substituted.)
2. Mordant: Copper sulphate, 20% aqueous solution.

Technique:

1. a. Exudate: Spread evenly on clean slide.
   b. Cultured organism: Mix with equal parts of animal serum and spread.
2. Air dry but do not heat fix.
3. Apply staining solution for 1 minute, heated to steaming.
4. Wash off the stain with copper sulphate. Do not wash with water.
5. Blot dry or examine wet under a cover slip.

Results: Capsule, if present, appears as a faint blue halo about a dark purple cell body.

11. Spore stain: (Dorner's method).

Formulae:
Carbol-fuchsin and Methylene blue as in acid-fast stain.

Technique:

1. Prepare slide spread and stain with carbol-fuchsin as for Ziehl-Neelsen method
2. Wash in hot tap water.
3. Rinse rapidly with 95% alcohol.
4. Apply Loeffler's Methylene blue for 2-3 minutes. Wash, blot dry.

Results: Spores are red, cell body blue.

12. Nigrosine method (for spirochetes)
Formulae:
Nigrosine-------------------10. gms.
Distilled water-------------90. cc.
Boil in flask for 30 minutes, then add as preservative:
Formalin (40%)--------------.5 cc.
Filter twice through double filter paper. Store in small sealed test tubes.

Technique: A loopful of fresh exudate or culture fluid is mixed on a slide with a loopful of nigrosine solution, then spread over the slide and dried.

Result: Spirochetes are not stained but are demonstrated negatively as unstained light areas on a smoky background.

13. Van Gieson's Stain (for Negri bodies).
Formulae:

1. Fixative:	Methanol (neutral) -------------	100. cc.	freshly prepared.
	Picric acid -------------------	.1 gm.	
2. Stain:	Basic fuchsin, stock solution -	.5 to 1 cc.	made fresh just before use. Fuchsin varied to desired result.
	Methylene blue, stock solution	10. cc.	
	Distilled water -------------	30. cc.	

Technique:
1. Make impression or smear spreads of grey matter of hippocampus or cerebellum of brain of suspected rabid animal.
2. Fix with a momentary flood of methanol. Wash at once.
3. Stain is applied for 5 minutes, heated gently to steaming. Wash, blot dry.

Result: Negri bodies are magenta with blue granules.
Nerve cells are blue.
Erythrocytes are salmon or bronze color.

14. Wright's stain and Giemsa's stain: See sections on Hematology and Parasitology.

Miscellaneous Solutions.

1. Sodium chloride solution ("Saline" or "physiological salt solution")
Sodium chloride ---------------------------------- 8.5 gms.
Distilled water ---------------------------------- 1000. cc.

2. Buffer solution:
Sodium dihydrogen phosphate (NaH_2PO_4) ---------- 28.81 gms
Disodium hydrogen phosphate (Na_2HPO_4) ----------125. gms.
Distilled water ---------------------------- to 1000. cc.

3. Sodium chloride solution, buffered:
Buffer solution (above) ------------------------ 20. cc.
Sodium chloride -------------------------------- 8.5 gms.
Distilled water ------------------------------to 1000. cc.

4. Sodium citrate - sodium chloride solutions: (anticoagulant)

	1%	2%	10%	
Sodium citrate c.p.	10. gms.	20. gms.	100. gms.)	filtered
Sodium chloride c.p.	8.5 gms.	8.5 gms.	8.5 gms.)	after
Distilled water		1000. cc.	1000. cc.)	solution.

May be sterilized in autoclave at 15 pounds for 20 minutes.
When used as anticoagulant of blood, a final sodium citrate concentration of over 0.25% is required; therefore, use to each 10 cc. of blood 3.3 cc. of the 1%, 1.4 cc. of the 2% or .26 cc. of the 10% solution.

5. Potassium oxalate solution: (anticoagulant)
Potassium oxalate ---------------- 2. gms.)
Sodium chloride ------------------- 6. gms.)1 cc. of this to 10
Distilled water -------------------100. cc.)cc. of blood prevents coagulation.

6. Sodium carbonate solution: 2 gms. per liter of water.
May be used for boiling instruments, as it prevents corrosion.

7. Disinfectant solution (Desk jar use)

	2%	5%
Liquor cresolis comp. U.S.P.	20.	50. cc.
Tap water to make	1000.	1000. cc.

8. Preparation of Percentage Solutions by Dilution:
   1. Measure number of cc. of more concentrated solution corresponding to the percentage desired for the new solution.
   2. Add distilled water to total volume corresponding to the percentage numeral of the original solution. For example, to prepare a 70% solution from a 95% solution, measure 70 cc. of the latter and add 25 cc. of distilled water, giving therefore 95 cc. of a 70% solution.

I. Introduction and Bacteriological Technique.

Bacteriology, strictly defined, is that branch of biology which treats of the structure and functions of certain minute plant forms called bacteria. However, the science of modern bacteriology, particularly medical bacteriology, has been enlarged to include other microorganisms such as certain fungi, plant forms of more complex structure; the spirochaetes, an indeterminate group variously classed as plants or animals by different investigators; the Rickettsia; and yet unclassified group of viruses.

Bacteria are intimately related to human existence. Without them higher animal and plant life would be impossible. Putrefaction and decay are dependent upon bacterial activity for the breakdown of complex compounds into simple forms which can be assimilated by higher plant life. If it were not for bacteria we would be lost in a mass of the debris of fallen leaves, dead animals, etc., which would not decay and return to the dust from which they came in their absence. Bacteria are also essential to the synthesis of nitrogen and carbon into compounds which can be utilized by plants and in turn by animals and man. The dependence of animal life upon plants completes the complex cycle of existence, in which bacteria play such a prominent role.

Unfortunately for us bacteria are not all our friends. Of the 1500 or more species which have been described, approximately 100 are pathogenic (cause disease) to animals and plants, and about 50 cause disease in man. It is with the pathogenic bacteria and related microorganisms that we are concerned in the laboratory.

Since these organisms are pathogenic, it is necessary to be careful in the laboratory when dealing with them for two reasons: (1) danger of infection to yourself and others, and (2) danger of contamination of the material to be studied. By contamination we mean mixing other microorganisms in with those to be studied. Since bacteria are everywhere present, in soil, water, air and on plants, animals and man, we have to use sterile technique in the laboratory to prevent contamination.

With regard to the danger of infection to yourself and others, when dealing with infected material, such as pus, sputum, spinal fluid, etc., do not spill it around on the table and chairs. Keep it off of your fingers, if possible, and above all, keep your fingers away from your face, mouth and eyes. When you are dealing with infected material, always think of your hands and fingers as if they had some of the material on them, even though you did not see any spill on your fingers; and keep your fingers away from your face until you have washed your hands thoroughly with soap and water. Frequent washing of the hands with plenty of soap and water never hurt anyone, but will save a lot of trouble.

A. Sterile Technique:

Sterile technique in bacteriology to prevent contamination is essential. In general this is accomplished by using alcohol

or gas flame, a platinum wire loop, and sterile glassware, and culture media. By sterile we mean the absence of living microorganisms. Sterilization is accomplished by holding glass or metal objects directly in a flame for about one minute, by boiling in water for 20 minutes or by steam or dry heat (see section on culture media for details of sterilization.) For the transfer of bacteria from a culture to a slide, or from one culture to another, or from the specimen to a slide or culture, the platinum wire loop is used. (The wire loop consists of a #22 or #24 platinum wire 3 or 4 inches long which is fixed to a handle of glass, metal or wood. The wire is straight except for a round loop at the far end about 4 millimeters in diameter). The wire is held in the flame almost perpendicular to the table until the entire wire is red hot, then, first allowing it to cool for a few seconds, the wire loop is placed in the culture or specimen and a small amount of the material is smeared on the slide. As soon as this transfer is made the wire loop is again flamed to red hot to remove the bacteria. <u>It is important to remember to flame the wire loop each time it is used</u>. If the procedure requires several transfers, flame the wire loop between each one.

B. Inoculating Cultures.

1. Broth Cultures.

   a. Of course the culture media must first be sterile and be in a sterile container (be it culture tube or flask) with a sterile plug.

   b. The inside of the container only is considered sterile - the outside is contaminated.

   c. Holding the culture container in the left hand, grasp the cotton plug with the hand inverted, between the fifth and four fingers of the right hand, then flame the mouth of the container. Holding the handle of the wire loop with the thumb and forefinger of the right hand (after first having flamed the wire loop), transfer a loop full of the material to the broth culture, being careful not to touch the outside of the container.

   d. Then hold the cotton plug in the flame until it burns and quickly replace it in the mouth of the container to put out the flame - never blow it out. Now flame the wire loop again before laying it aside.

   e. If you are to make a blood culture, the blood will be collected with a sterile syringe and needle (3 or 4 cc. of blood is sufficient). Now steps a, b, c, and d are followed except that instead of using the wire loop for the transfer, the sterile needle of the syringe is placed inside the mouth of the container (being careful not to touch the outside with the needle), and the blood is forced into the culture by holding the barrel of the syringe between the index and middle fingers of the right hand and pushing down the plunger with the right thumb.

2. Agar Cultures.
   a. Slant agar - the same procedure is followed as for broth cultures. The transfer is made with the wire loop but instead of dipping the loop into the culture it is rubbed lightly over the surface of the slant.
   b. Stab agar cultures - the same procedure again except instead of a wire loop a straight wire without a loop is used and this is stabbed into the agar culture.
3. Plate Cultures: plate cultures are made by placing sterile, melted culture media in sterile Petri dishes and, after allowing an initial incubation period to test their sterility, they may be stored in a refrigerator until needed.
   a. Streaked Plate Method: tubes containing 10-15 cc. of sterile agar medium are held in boiling water until the agar is melted. Then allow it to cool until the tube can be handled. Now holding the tube in the right hand, remove the cotton plug with the inverted left hand, grasping the plug between the fifth and fourth fingers. Flame the mouth of the tube, then lift one edge of the cover of a sterile Petri dish just high enough to pour the melted agar into it, quickly replace the cover of the Petri dish and allow the agar to solidify evenly distributed over the plate. After the plate has hardened or set, it is incubated at 37.5° C. for 12 hours to check its sterility. A large number of culture plates may be prepared in this way and then stored in a refrigerator until needed.
      (1) Inoculation of Streaked Plate: this can be done with a sterile swab or a wire loop. One edge of the cover is lifted just high enough to admit the swab or wire loop and this is drawn over the surface in parallel rows 4-6 mm. apart without recharging the loop, then quickly replace the cover. Now invert the Petri dish and mark it with a wax pencil. The plate cultures are then incubated in an inverted position to prevent the spread of the colonies on the surface by the water of condensation.
   b. Poured Plate Method:
      (1) Plain agar poured plate - serial dilutions of a mixed culture are made in tubes of melted agar. Usually three dilutions are sufficient to obtain isolated colonies. One or two wire loopsful of the mixed culture are put into the first tube of melted and cooled agar. The tube is rolled between the hands to insure thorough mixing without the formation of bubbles. Then two loopsful are transferred from this tube to a second cooled agar tube and, after thorough mixing, three loopsful from the second to the

third tube. The contents of each tube are then poured into a sterile Petri dish, on the underside of which the date and other necessary information has been marked with a wax pencil. The work must be carried out rapidly in order to be completed before the agar solidifies.

(2) Blood Agar Pour Plates: by adding sterile blood (sheep, rabbit, or human) to the medium, blood agar for determining the type of hemolysis produced by certain bacteria may be made. Hemolysis is best determined by the use of poured plates, although for convenience in general laboratory work the blood agar is allowed to solidify and then inoculated by streaking.

II. Types of Bacteria.

A. Morphology: by morphology we mean the size, shape and general structural form of the bacteria. There are three general forms.

1. Cocci - cocci, when lying free, are approximately spherical like a ball. Variations from the strictly spherical shape are of common occurrence. The crowded conditions of rapid growth often give a variety of ellipsoidal, conical and flattened forms, popularly designated as bean, kidney and lancet-shaped. Division may occur in one direction resulting in the formation of diplococci and chains; in two directions in a single plane at right angles to each other; in three directions, producing the cubical packets of sarcina; and irregularly in all three directions, giving the clustered formation of the staphylococci. In addition the cocci may have capsules. The most important of these encapsulated cocci is the pneumococcus.

2. Bacilli - the Bacilli, which include the greatest number of species, are straight, cylindrical, rod-shaped cells, with plane, convex, or concave ends. Although they are uniformly longer than wide, the ratio of length to width varies greatly, some forms being filamentous and others approaching the coccal shape. Variations such as ovals, spindles and club or dumb-bell shaped forms occur. Morphological characteristics such as size, ratio of length to width, and contour of the ends, serve to identify a few species, but final confirmation requires cultural and staining methods. Division always occurs at right angles to the longitudinal axis.

3. Spirilla - the spirillar type is characterized by a bending or twisting which produces a curved, helical or spring-like shape. The spirilla vary from small comma-shaped forms (vibrio) with a single turn, representing the segment of a helix, to long sinuous forms with several curves. In this class the spirochaete of syphilis may be included. The organism variously called Borellia Vincenti, Spirochaete of Vincent's, can also be included here. This organism, which causes Vincent's Angina, is usually found in two forms, one a spindle-shaped or cigar-shaped form and the other a wavy spirillum.

B. Motility: some bacteria, mostly bacilli and the spirilla, have the power of motion. This power of motion is used as another aid in the classification of the bacteria. The bacteria move in their liquid environment by moving their flagella. The flagella are very fine projections of the bacteria situated at the ends or along the body of the organism. The flagella are so small that they cannot be seen by ordinary methods, however, in wet preparations, if the bacteria are motile, it is assumed that they have flagella. By motility of bacteria we do not mean the vibration back and forth called Brownian Movement, we mean active motion about the microscopic field.

C. Spores: when conditions for bacterial existence become unfavorable, some species develop spores, resting forms possessing greater powers of resistance than those of vegetative bacteria. These spores are known as endospores or true spores to differentiate them from the sexual and asexual reproductive spores of fungi. They appear in the body of the bacteria as highly refractive round or oval bodies, which are resistant to ordinary dyes and require special staining methods. The mature spore consists of condensed nuclear and cytoplasmic material enclosed in a relatively impenetrable spore wall. With the completion of the fully-formed spore, the parent cell disintegrates. When the conditions are favorable the spore is capable of developing into the vegetative form of the bacterium.

The presence or absence of spores is another aid in the classification of bacteria. Since the spores are very heat resistant, high temperatures are necessary to destroy them.

D. Cultural Characteristics: the kind of culture media that the organism grows best on; (some will grow only on special media) the color, size, shape, and elevation of the colonies; whether they will grow in the presence of oxygen (aerobic) or will grow only in the absence of oxygen (anaerobic) all are aids in the classification of the kind of bacteria.

E. Fermentation of Sugars: certain of the bacteria will ferment or produce gas in various kinds of carbohydrates such as: indol, dextrose, maltose, mannite, sucrose and lactose. Some organisms will produce acids when grown in the above carbohydrates and, by the addition of an indicator, this can be determined by the color change. All of this is important in the identification of the various types of bacteria. (See the Bacteriology Reaction Chart).

ORGANISM	FORM	Gram's	Spores	Motility	Indol	AGAR SLANT	BROTH	PEPTONE	HABITAT	REMARKS AND DIAGNOSTIC CHARACT.
Staph. Albus	Spherical	+	−	−	−	Abundant Shiny, White	Turbid, pellicle, Sediment	Sediment	Skin, air, etc.	White, Grape-like Clusters
Staph. Aureus	Spherical	+	−	−	−	Ab. Sh. Orange	Tur. Sed.	Sediment	Skin, air, etc.	Orange, Grape-like Clusters
Strep. Pyogenes	Pairs or Chains	+	−	−	−	Scant Colonies	Scant	Scant	Skin Pathogenic	Hemol. on bl. agar
Strep. Viridans	Pairs or Chains	+	−	−	−	Scant Colonies	Scant	Scant	Mouth Intestine	Green Zone on bl. agar
Diplo. pneumoniae	Pairs or Chains	+	−	−	−	Scant Colonies	Scant	Scant	Mouth, Nose	Capsules, Bile Soluble
Meningococcus	Pairs	−	−	−	−	Tiny or none	Scant	Scant	Meningitis Nose	Growth best on Blood Media
Gonococcus	Pairs	−	−	−	−	No Growth	No Growth	No Growth	Gonorrhea	Growth only on Special Media
Esch. Coli	Short Rods	−	−	+	+	Moist, opaque Fecal Odor	Turbid Sed.	Turbid Sed.	Intestine	Ferment Sugars, Methyl Red
Salmonella paratyphi	Rods	−	−	+	−	Moist, Gray	Turbid Sed.	Turbid Sec.	Paratyphoid	Fermented Sugars, H_2S not formed
Salmonella schottmulleri	Rods	−	−	+	−	Moist, Gray	Turbid Sed.	Turbid Sed.	Food poisoning, paratyph.	Ferment sugars H_2S Formed
Shegella dysenteriae	Short Rods	−	−	−	−	Moist, Gray	Sl. Turbid	Sl. Turbid	Dysentery	Ferment sugars, exotoxin produced
Sheg. paradysenteriae	Short Rods	−	−	−	+	Moist, Gray	Turbid	Turbid	Dysentery	Ferment Sugars, Indol +
B. anthracis	Square End Rods	+	+	−	−	Gray Spreading	Pellicle Sed.	Pellicle Sed.	Causes Anthrax	In tissue as encapsulated chains
Clos. tetani	Large Rods	+	+	+		Small, Gray	Sl. Turbid	Sl. Turbid	Soil, Tetanus	Anaerobic Toxin produced
Clos. welchii	Large Rods	+	+	−	−	Moist, Gray	Turbid	Turbid	Soil, Gas Gangrene	Anaerobic Toxin Produced
Clos. botulinum	Large Rods	+	+	+		Flat, Gray	Turbid	Turbid	Soil, Botulism	Anaerobic Toxin Produced
Tubercle bacillus	Slender Rods	+	−	−		No Growth	No Growth	No Growth	Human Pathogen	Acid Fast, grows in guinea pig
Diphtheria bacillus	Rods	+	−	−	−	scant gray, granular	Sl. Turbid	Sl. Turbid	Nose & throat Diphtheria	Grows on Loeffler's bl. serum, Club shaped, beaded
Eberthella Typhosa	Short Rods	−	−	+	−	Moist, gray	Turbid	Turbid	Typhoid fever	Ferment Sugars, Motile
B. Vincenti	Fusiform Spirillum	Stains with Wright's or dilute carbol fuschin							Mouth & throat V. Angina	Cigar-shaped 4–8 µ Wavy threads 10–20 µ

Cutline Classification of Bacteria of Greatest Importance in Medical Bacteriology, due to their Pathogenicity or to their Frequent presence in Cultures as Contaminants or Saprophytes.

I. COCCI. Cells spherical or somewhat elliptical. Aerobic.

A. Gram Positive

1. Streptococcus forms. Cells in short or long chains, never in packets. Genus Streptococcus.
   a. Pyogenic group. Generally (beta) hemolytic. Four species several serological types. Most important species - S.pyogenes.
   b. Viridans group. Not beta hemolytic but may show varying degrees of greening (alpha) of blood. Most important species - S.salivarius
   c. Saprophytic group. No hemolysis or only indistinct zone (alpha prime type). Usually not pathogenic. Example - S.faecalis

2. Diplococcus forms. Cells usually in pairs. No hemolysis. Colonies greenish on blood agar. Diplococcus pneumoniae.

3. Micrococcus forms. Cells in plates, groups or irregular packets or masses. Never in chains. Usually non-pathogenic. Genus Micrococcus.

4. Staphylococcus forms. Cells as a rule in irregular groups. Usually pathogenic. Genus Staphylococcus.
   a. Orange pigment. Staph. aureus.
   b. Lemon-yellow pigment. Staph. citreus.
   c. White or colorless growth on solid media. Staph. albus.

5. Tetragena forms. Occurs in pairs and tetrads. Gaffkya tetragena.

6. Sarcina forms. Division occurs in three planes, producing regular packets. Example - Sarcina lutea.

B. Gram Negative

1. Cells normally in pairs, with adjacent sides usually flattened. Genus Neisseria.
   a. Grow best on special culture media at 37°C.
      (1) Acid from dextrose, not from maltose. N.gonorrhoea.
      (2) Acid from dextrose and maltose. N.intracellularis.
   b. Grow well on ordinary culture media at 22°C. Usually not pathogenic. Several species.
      Examples: (1) Non-chromogenic - N. catarrhalis.
      (2) Chromogenic - N.flava.

II. CURVED FORMS. Cells elongate, more or less spirally curved, Gram-negative. Vibrio comma.

III. NON-SPORULATING BACILLI. Elongated rod-shaped cells, without endospores. Gram-positive or Gram-negative. Aerobic

A. Gram-positive.

1. Long slender, non-motile rods, occurring singly in pairs and in chains. Genus Lactobacillus.

B. Gram negative.

1. Small, motile or non-motile rods. Not active in fermentation of carbohydrates. Usually parasitic on warm blooded animals. Frequently require body fluids for growth. (Family Parvobacteriaceae).

a. Majority grow on ordinary media. Show bi-polar staining. Majority ferment carbohydrates.
Tribe Pasteurelleae - Genus pasteurella.
(1) Grow on ordinary media. Indol and H_2S produced. No growth in bile. Sorbitol fermented. Animal pasteurellas (5 species).
(2) Grow on ordinary media. Neither indol nor H_2S produced. Growth in bile. Sorbitol not fermented. Pasteurella pestis.
(3) No growth on ordinary media. Pasteurella tularensis.

b. Majority grow on ordinary media. Do not show bi-polar staining. None ferment carbohydrates. Non-motile. Three species of genus Brucella:

	Growth in media containing:	
	Thionin	Basic fuchsin
Br.melitensis	+ + +	+ + +
Br.abortus	- - -	+ + +
Br.suis	+ + +	- - -

c. On first isolation require some factor or factors contained in blood or plant tissues. Usually do not show bi-polar staining. Genus Hemophilus.
(1) X and V factors required - H.influenzae.
(2) Neither X nor V factors required - H.pertussis and H.ducreyi.

2. Motile or non-motile rods widely distributed in nature. Majority of species attack carbohydrates forming acid, or acid and gas. Grow well on artificial media.

a. Do not produce acid in media containing carbohydrates.
(1) Rounded colony, no pigment produced. Alcaligenes facealis.
(2) Large spreading colony, yellowish-green pigment produced. Pseudomonas aeruginosa.

b. Ferment dextrose and sucrose but not lactose with formation of acid and small amount of gas. Produce characteristic spreading, amoeboid colonies. Liquefy gelatin.

<u>Proteus vulgaris</u>.

c. Ferment dextrose and lactose with formation of acid and gas. So called coli-aerogenes group.

Species	Methyl red	V.P.	Citrate utilization	Indol	Gelatin liquefaction
<u>Escherichia coli</u>	+	-	-	+	-
<u>Escherichia freundii</u>	+	(?)	+	±	?
<u>Aerobacter aerogenes</u>	-	+	+	?	-
<u>Aerobacter cloacae</u>	-	+	+	-	+
<u>Klebsiella pneumoniae</u>	+	-	+	-	-

d. Ferment dextrose with formation of acid, or acid and gas. A few species of genus <u>Shigella</u> ferment lactose with formation of acid, but never visible gas. Tribe <u>Salmonelleae</u>.
(1) Ferment dextrose with formation of acid and gas .
Genus <u>Salmonella.</u>
(2) Ferment dextrose with the formation of acid but no gas.
(a) Motile - <u>Eberthella typhosa</u>.
(b) Non-morile - Genus <u>Shigella</u>.

Salmonella - typhoid-dysentery group

	Dextrose	Mannitol	Maltose	Xylose	Dulcitol	Lactose	Sacchrose	Inositol	Indol	H_2S production	Citrate utilization	d-tartrate utilization	Motility
Salmonella choleraesuis	AG	AG	AG	AG	(AG)	-	-	-	-	-	+	+	+
Salmonella pullorum	AG	AG	-	(AG)	-	-	-	-	-	+		-	-
Salmonella paratyphi	AG	AG	AG	-	(AG)	-	-	-	-	(+)	-	-	+
Salmonella enteritidis	AG	AG	AG	AG	AG	-	-	-	-	+	+	+	+
Salmonella schottmuelleri	AG	AG	AG	(AG)	(AG)	-	-	(AG)	-	+	+	-	+
Salmonella typhimurium	AG	AG	AG	AG	AG	-	-	(AG)	-	+	+	+	+
Eberthella typhosa	A	A	A	(A)	(A)	-	-	-	-	+	-	+	+
Shigella dysenteriae	A	-	-	-	-	-	-	-	-	-	-		-
Shigella paradysenteriae	A	A	(A)	-	-	-	(A)		+	-	-		-
Shigella alkalescens	A	A	A	A	A	-	(A)		+		-		-
Shigella sonnei	A	A	A	-	-	A	A	-	-		-		-
Shigella madampensis	A	A	A	A	-	A	A	-	+	-	-		-

Note: Brackets () around symbol denotes variable or delayed reaction.

IV. <u>SPORULATING BACILLI</u>. Rods producing endospores, usually Gram-positive. Often decompose protein media actively.

A. <u>Grow aerobically</u>. Mostly saprophytes. Genus <u>Bacillus</u>.

1. Pathogenic forms. Non-motile rods with square cut to concave ends, occurring in long chains, central spores.
<u>Bacillus anthracis</u>.

2. Non-pathogenic forms. Usually motile, having central or excentric spores. <u>B.subtilis</u> group (145 species)

B. Grow only anaerobically. Often parasitic. Genus <u>Clostridium</u>.

1. Non-motile rods. Rods not swollen at sporulation. Spores central or excentric. <u>Cl.perfringens</u>.

2. Motile. Rods swollen at sporulation.
   a. Spores terminal or subterminal. Spherical or nearly so. <u>Cl.tetani</u>.
   b. Spores oval, central or excentric.
      (1) Pathogenic to man - due to preformed toxin.
         (a) <u>Cl.parabotulinum</u>.
         (b) <u>Cl.botulinum</u>.
      (2) Pathogenic to man - associated with gas gangrene.
         (a) <u>Cl.novyi</u>
         (b) <u>Cl.septicum</u>
         (c) <u>Cl.bifermentans</u>
         (d) <u>Cl.histolyticum</u>
         (e) <u>Cl.fallax</u>
      (3) Not pathogenic to man - many species. Examples:
         (a) <u>Cl.sporogenes</u>
         (b) <u>Cl.tertium</u>

V. <u>BACILLI HAVING BRANCHING CHARACTERISTICS</u>. Show parallelism, slight branching, curving forms, V-shapes, clubbing at ends, and segmental staining. Gram-positive.

1. Not acid fast. Colonies more flat and moist like other bacteria. Rods frequently club shaped. Genus <u>Corynebacterium</u>.

   a. True diphtheria organism. Slender rods, curved or straight, of variable lengths; granular or segmented; generally club shaped. Metachromatic granules large except in gravis type. Moderate growth on ordinary media. <u>C.diphtheriae</u>.

   b. The diphtheroid group of bacteria - 20 species.
      (1) Short, thick, straight rods. Stain uniformally. Luxuriant growth on ordinary media. <u>C.pseudodiphthericum</u>

      (2) Medium sized rods showing solid and barred forms. Metachromatic granules small. Scanty and slow growth on ordinary media. <u>C.xerose</u>.

Species	Dextrose	Maltose	Dextrin	Glycerol	Galactose	Saccharose	Litmus milk	Production of an exotoxin	Production of hemolysis
C.diphtheriae Type I	+	+	+	-	-	-	-	+	+
" Type II	+	-	-	-	-	-	-	+	+
" Types III,IV,V	+	+	+	+	+	-	-	+	+
C.pseudodiphthericum	-	-	-	-	-	-	-	-	-
C.xerose	+	+	-	-	+	+	-	-	-
C.segmentosum	+	+	-	+	-	±		-	-

2. Acid fast. Colonies more or less wrinkled and dry, more like moulds. Slender rods, seldom filaments, which are stained with difficulty, but when once stained are acid fast. Cells sometimes show swollen, clavate or cuneate forms and sometimes even branched forms. Genus Mycobacterium.

   a. Saprophytes, or parasites on cold blooded animals; grow rapidly on most media at room temperature. (8 species).
      Examples: (1) M.lacticola.
      (2) M.phlei

   b. Parasites on warm blooded animals; grow slowly on all media.
      (1) Pathogenic for man.
         (a) M.tuberculosis var. hominis.
         (b) M.tuberculosis var. bovis.

      (2) Not pathogenic for man. M.avium.

   c. Pathogenic for man. Will not grow on usual culture media. M.leprae.

GENUS STREPTOCOCCUS

Habitat: Common pathogenic forms; also frequently on skin and body orifices without invasive tendency. Some species are the specific causes of infectious diseases. A number of saprophytic species are commonly present in dairy products and elsewhere.

Characteristics: Gram positive cocci of medium size, in pairs or short chains, never in packets; grow best on blood or serum agar, aerobically, at 37°C., the 24 hour colony being small, circular, slightly raised, surrounded at times by zone of haemolysis. Killed at 55°C., in 30 minutes.

Haemolytic Group (Beta type) have clear zone of haemolysis around colony on blood agar.

Viridans Group (Alpha type) have greenish zone around colony on blood agar.

Non-haemolytic Group (Gamma type) have no area of haemolysis or green zone around colony.

Streptococcus pyogenes: Colonies have Beta zone of haemolysis 2 to 3 mm. wide. Grow in long chains. Found in man in acute inflammations including septicaemia, cellulitis, wound infections, middle ear or sinus disease or elsewhere. Tend to be more severe and generalized than Staph. aureus infections.

Streptococcus salivarious: (S. viridans) (S. mitior) is a parasite of the normal nose and throat, also encountered in dental abscesses, in endocarditis and in some blood cultures. Grow in short chains. This colony readily recognized on a blood agar plate by its greenish zone of haemolysis. Usually not pathogenic for small animals. Distinguished from Diplococcus pneumoniae by inability to ferment inulin and by not being bile soluble.

Streptococcus lactis: Is non-pathogenic, occurs in milk and milk products and in mouth and intestinal tract of man. Colonies on blood plates preduce no haemolysis or only trace of green.

Streptococcus faecalis: Is feebly pathogenic, found in feces of man and other animals. Sometimes found in inflammatory exudates and subacute endocarditis. No haemolysis on blood agar.

Identification:

1. Microscopic: Gram stain of direct or culture spreads will show Gram ≠ cocci, singly, in pairs or in chains of varying length. The chain form is best seen in spreads made from liquid culture, or in liquid body fluids.

2. Culture: Blood agar plates at 37° C. for 24 hours will give the small colony type and form of haemolysis classifying roughly the species. For routine clinical work the examination is usually limited to the study of colonies on blood agar and the results reported as the case may be:

Streptococcus, haemolytic
" non-haemolytic
" viridans

GENUS STAPHYLOCOCCUS

Habitat: Common, potential or actual parasites, occurring on normal skin and body orifices, and in feces, therefore in dust, soils and as culture contaminants; frequently the cause of suppurative lesions in man.

Characteristics: Moderate size cocci, are in pairs or grape-like clusters; gram-positive; grow freely, aerobically, on common culture media, giving in 24 hrs. at 37° C. medium size, low, convex, smooth, glistening colonies with an even edge; color of colony variable with species; some strains produce haemolysis on blood agar.

Staphylococcus aureus: Golden yellow colony; usually haemolytic; frequently found in boils, carbuncles and other skin lesions; sometimes in blood cultures in the event of septicaemia.

Staphylococcus albus: Porcelain-white colony; feebly pathogenic.

Staphylococcus citreus: Lemon-yellow colony. A non-pathogenic saprophyte.

Identification:

1. Microscopic: Gram ≠ staphylococci on direct or culture stained spread.

2. Culture: blood agar plate, 24 hrs. at 37° C. gives colony features of staphylococcus, species determined by color of colony. Note also presence or absence of haemolysis.

DIPLOCOCCUS PNEUMONIAE (Pneumococcus)

Characteristics: Large lancet shaped cocci, usually occurring in pairs; sometimes found singly or in short chains. When in pairs, the adjacent ends of the cocci are usually bluntly rounded, the opposite ends are acutely pointed. In films from sputum, blood and cultures on serum containing media, a definite capsule can be seen. Gram-positive; stains well with aniline stains and special capsule stains. Poor growth on plain agar; grows best on blood or serum agar with pH 7.6 to 7.8; colonies on blood agar plate, surface flat and smooth with edge sharply raised from the medium, surrounded by a narrow zone of alpha-haemolysis (green discoloration); some strains (Types III and VIII) give characteristic mucoid colonies. Killed in 20 minutes or less at 55°C. Bile soluble; ferments inulin. 31 distinct serological types have been identified; called D. pneumoniae Type I, II, etc. to XXXIII; however, types 26 and 30 apparently are identical with types 6 and 15, respectively.

Habitat: The principal cause of lobar pneumonia (over 90%); also may cause bronchitis; bronchopneumonia, conjunctivitis, otitis media, brain abscess, meningitis, endocarditis and arthritis. Frequently present in normal mouths. Highly pathogenic for mice and slightly less so for rabbits.

Identification:

1. Direct microscopy: make spreads of specimen on slide, fix and stain by Gram's method and/or Hiss's capsule stain. Examine for diplococci showing typical morphology; if present confirm by procedures below.

2. Typing by Neufeld Reaction: This is the rapid method of choice for identification of type on materials direct from the patient, giving the type within 30 minutes. It is less applicable to typing of cultures or to detection of type in patients who have received one of the sulphanilamide compounds. Only after pneumococci have been shown by stained spread to be present in appreciable numbers, is this typing effort to be attempted.

LABORATORY DIAGNOSIS OF PNEUMOCOCCUS PNEUMONIA

The treatment of penumonia with type specific therapeutic serum has increased in recent years. Coincident with this, the Neufeld reaction has assumed a greater significance, since specific treatment cannot be administered without knowledge of the type of pneumococcus causing the infection. The more recent introduction of sulfapyridine, and other sulfone derivatives, as a chemical means of treating pneumonia has not abolished the desirability of the bacterial method of control. In order to have a comprehensive treatment, it is important to know the kind of organism. Even if chemical therapy alone is to be used, it is necessary to know the type of the causative pneumococcus since certain types require different dosages of the drug. Furthermore, for statistical purposes in order to develop rational methods for the prevention and control of pneumonia, it is necessary to determine the infective agent in every case.

Sputum

Although pneumococci from any source can be typed, for usual diagnostic purposes in pneumonia, sputum is most satisfactory. It should be collected in clean, dry and preferably sterile wide-mouthed containers. Since the determination of the type may depend on the presence of living organisms, no antiseptic should be added. If the specimen is to be transported any distance requiring more than 5 hours, and cannot be kept cold, formalin (0.5%) may be added as a preservative. This will not interfere with the test. Generally the sputum obtained from pneumonia patients contains some blood and tends to be thick and sticky, or may be the color and consistency of prune juice. It is not necessary to have a large amount of sputum; whenever possible, at least one or two teaspoonsfull should be obtained.

The sputum should be fresh and should be examined soon after expectoration by the patient. If there is a delay in the examination, the specimen should be kept in a refrigerator to retard the overgrowth of other organisms. Pneumococcus typing should be considered as an emergency, and the report should be submitted as soon as the results will permit.

Blood Cultures

Blood cultures are made by mixing two or three cubic centimeters of blood with 50 cc. or more of broth, and observed after eight to twelve hours of incubation at 37.5°. If bacterial growth is not evident, incubation is continued and the culture observed at intervals for at least 72 hours, and preferably 7 days. In positive broth blood cultures, the supernatant fluid shows a diffuse turbidity and on agitation the mixture appears brownish-red to chocolate in color. Usually a sufficient number of organisms is present, as shown

by a gram-stained smear of the culture, so that the Neufeld technic may be carried out directly. If growth is sparse, it is advisable to place 10 cc of the culture aseptically into a sterile tube and centrifuge at low speed to remove the blood cells. The supernatant fluid is poured into a second tube and centrifuged at a high speed to throw down the organisms. The sediment of organisms is suspended in a small amount of broth for typing.

Serums

In the Neufeld test, highly type-specific antiserum should be used which are prepared in rabbits especially for this reaction. Neither therapeutic nor diagnostic agglutinating horse antiserums are satisfactory because of occasional non-specific reactions.

There are now some 31 different recognized types of pneumococci. In order to shorten the procedure varying numbers of these are mixed together into 6 different combinations of from 3 to 6 types in each. These 6 combinations are lettered from A to F inclusive. The specimen is first tested against each of the 6 combinations. If a positive result is obtained in one of them, then the specimen is tested against each of the component types of that combination. These now include:

Group	A	- - Type	1, 2, 7
"	B	- - "	3, 4, 5, 6, 8
"	C	- - "	9, 12, 14, 15, 17, 33
"	D	- - "	10, 11, 13, 20, 22, 24
"	E	- - "	16, 18, 19, 21, 28
"	F	- - "	23, 25, 27, 29, 31, 32

It is not necessary to try to memorize these combinations since they are furnished with each diagnostic set and the various component types are on the label of the vials. When not in use the serums should be kept in the refrigerators. Care also should be exercised to prevent their contamination.

Neufeld Reaction - Procedure

The Neufeld "quelling" reaction is based on the interaction between the encapsulated organism and the specific homologous antibody in the antiserum, resulting in the apparent swelling of the capsule without obvious change in size of the organism within.

Wet Preparation Method

1. Areas are marked off and labeled on clean glass slides for the group typing serums.

2. By means of a small loop measuring approximately 1 mm. in diameter, a small amount of the specimen is placed on each area of the slides.

3. With a large loop measuring 4 mm. in diameter, approximately ten times the amount of undilated serum is mixed with the material according to the relative number of organisms present; a different type serum or group of serums is used for each area. The loop should be cooled and flamed between each transfer.

4. Loeffler's methylene blue is next added with the 1 m.m. loop and mixed with the specimen and serum. Some typing serums already may contain this dye, but it is often insufficient for staining.

5. A cover-slip is placed over each preparation. The slides are allowed to stand for a few minutes before examination.

Examination

A good microscope with an oil immersion lens is essential and a powerful microscope light, subdued with blue glass, is a great aid.

The entire preparation should be examined with an oil immersion lens. This test is highly specific when satisfactory antiserums are used. If no reaction occurs immediately, the preparation should be examined again in about 30 minutes.

In a preparation containing the homologous antiserum, a positive reaction is indicated by the appearance of a definitely outlined but colorless halo surrounding the blue-stained pneumococci. This zone is swollen capsular material, having a distinct "ground glass" appearance. It is not the size, but the typical appearance of the swollen capsule which determines the positive reaction. The degree of swelling is usually equivalent to the width of the pneumococcus, although that of Type III. is much greater. In most types, the sharpness of outline is most significant. In some negative reactions, a thin halo without definite outline may confuse the inexperienced observer. Focusing just above and below the pneumococcus itself, reveals nothing in such preparations; whereas, in positive reactions the swelling is easily visible at these focal planes. When a positive reaction is found in one of the 6 groups, then the same process is repeated for each of the specific types composing that group.

Sources of Error Involved in the Neufeld Test

1. Proportion of serum to sputum.-This is one of the most common causes of failure to obtain a reaction. Satisfactory ratio of serum to sputum should be more than 5 to 1. If the specimen contains very large numbers of pneumococci, it should be diluted with broth or saline.

2. Influence of thick tenacious sputum. - Thick tenacious sputum will retard or prevent the reaction entirely. In addition, there is great difficulty in picking up a small portion of sputum. To avoid

this thick sputum should be mixed with a small quantity of saline or broth and beaten with a wooden applicator, thus releasing sufficient numburs of the organisms.

3. Specimens containing two or more types. - This may occur and for this reason it is necessary to examine all six of the groups. When such a case is found, it is necessary to make a note as to which specific type is the most numerous; since the type which is most numerous is probably the causative organism. Types III, VI, XIX, and XXIII, are relatively more common in normal throats than any other types, and may be found along with the causative organism. Types I and II are rarely found in normal throats.

Mouse Inoculation

Specimens containing pneumococci which fail to type by the Neufeld reaction should be examined further by inoculation into a blood agar plate and by intraperitoneal injection into a mouse. This will demonstrate the presence of pneumococci which were overlooked by the direct examination. About 0.5 cc. of the material is injected into the peritoneum of a mouse, and the animal observed at frequent intervals for signs of illness. Symptoms may occur after 4 or 5 hours. At that time, or after 18 hours, a puncture of the peritoneum is made. This is done with a capillary pipette (75 mm..long by 1 mm. in diameter) or with a tuberculin syringe and a 25 gauge needle. The material thus obtained is examined by the Neufeld test, care being taken that only small amounts of the exudate is used. If the mouse dies the abdomen is opened and the exudate examined in the usual manner.

Bile Solubility Test

The solubility of the pneumococcus in bile affords one of the most satisfactory tests for distinguishing pneumococci from streptococci. Broth cultures 18 to 24 hours old are used for this test. Into a small precipitin tube 0.2 cc. of the culture is placed, to which is added 0.1 cc. of ox-bile. A control tube is set up, using a similar amount of culture and 0.1 cc. of saline. Another control is generally set up for clarity and color to be expected, mixing 0.2 cc. of plain broth with 0.1 cc. of ox-bile. The tubes are incubated in a water bath at 45° C., for 1 hour or at 37° C. for 2 hours and then examined. Those cultures which lyse (all bacteria dissolved) completely in bile are soluble, and are therefore pneumococci.

Bile for this purpose must of necessity be crystal clear. Centrifugation may clarify it, although filtration is sometimes necessary. A 10% solution of bile salts (sodium desoxycholate or taurocholate) may be substituted for bile. Each new lot of bile or bile salts should be first tested with known pneumococcus cultures.

NEISSERIA

Characteristics: Gram-negative cocci of variable growth vigor and variable pathogenicity. All members give a positive oxydase reaction.

Habitat: N. gonorrhoeae (gonococcus) is the cause of gonorrhea; N. intracellularis (meningococcus) is the cause of a specific meningitis; both organisms may be readily demonstrated in the exudates from involved tissues; N. catarrhalis and several other species, which are found in the nose and throat of normal individuals, are sometimes associated with certain epidemics of respiratory or eye infections.

NEISSERIA GONORRHOEAE (gonococcus): A strict parasite of man; found in discharges from the genito-urinary system in acute or chronic gonorrhea, in the pus from gonorrhoeal conjunctivitis, rarely in the blood stream.
Characteristics: Oval or spherical cocci of moderate size, frequently arranged in pairs with adjacent sides flattened or slightly concave, resembling a pair of kidney beans side by side; in exudates the cocci are fairly regular in size and shape and are usually inside the pus cells; in cultures the cocci will not grow on plain agar, enrichment of media is needed; grow on moist chocolate agar at 37° C. in 24 hours to small, round, convex, greyish-white colonies; growth is aerobic, favored by atmosphere of 10% CO_2. Highly susceptible to inimical agencies: when dried the cocci die in 2 hours; moist heat at 55°C. kills in 5 minutes; quickly killed by 1:4000 silver nitrate; cultures kept at room temperature die in a few days, but at 37°C. they may survive several weeks.
Identification: Microscopic examination only is generally done, cultural confirmation done only under special conditions.
1. Microscopic: Make direct spreads of the infected urethral, cervical or conjunctival discharges on glass slides, fix with heat and stain by Gram's method. Examine the stained preparation for gram-negative, coffee bean shaped, intracellular or extracellular diplococci having the typical morphology of gonococci. Report whether diplococci are intra or extra cellular, or both. Also report any other bacterial forms present, noting for each whether Gram-negative or Gram-positive and whether coccus or bacillus; also the relative numbers and kinds of tissue cells present.

2. Ordinary culture methods, especially in chronic urethral or cervical infections, will reveal only the secondary organisms which may occur. A special culture program is needed for growing N. gonorrhoeae.
3. Special culture program: The cultural demonstration of the gonococcus is superior to direct spread examinations in cases of chronic gonorrhoeae in both sexes and in all cases in the female, especially when material for examination is taken from the cervix.

a. The cultivation of the gonococcus, mixed with freer growing micro-organisms, requires observance of the following special procedures:

(1) Take specimens of representative material and apply directly to plate media.

(2) Use a medium such as moist chocolate agar, which will readily grow the gonococcus in mixed culture.

(3) Grow in 10% CO_2.

(4) Identify the gonococcus-meningococcus group by colony form and oxydase reaction.

(5) Confirm the identification carbohydrate fermentation tests.

b. Specimen taking and transmission: (Optional methods listed in order of preference.)

(1) Platinum loop is touched to drop of pus, to urethra or to cleansed cervical os, and is immediately stroked broadly over a warm culture plate at the bedside or clinic chair.

(2) Sterile swab is similarly contaminated with the suspected material at the bedside or clinic chair, immediately placed in a tube containing 1 cc. of nutrient broth for prompt transmission to laboratory and inoculation of warm culture plate (broad spread of .1 cc. of this broth).

(3) For delayed inoculation (up to 8 hours), the swab-broth tube #2 is stored in icebox until culture plate inoculation is made.

c. Culture Media: (1) chocolate agar, soft, moist, warm.

(2) The media of McLeod is elsewhere described.

(3) Difco "Proteose #3 Agar" and "Bacto Hemoglobin" may be combined.

d. Incubation: 37° C. in 10% CO_2 in closed jar, 24 - 48 hours.

e. Examination of Culture: Observation made of two features:

(1) Colony form: convex, slightly opague colonies, 1-3 mm. in diameter, with undulated margins. Their slight opacity and characteristic undulated margins serve to differentiate them from colonies of streptococci and of diphtheroids.

(2) Oxydase reaction: Flood a segment of the plate with 1 cc. of 1% aqueous solution of dimethyl paraphenylene diamine hydrochloride (Eastman Kodak Co.). (The McLeod program similarly uses 1% tetramethyl paraphenylene diamine hydrochloride, giving the colonies a bright purple color, is more expensive but has the advantage of a more rapid reaction and not killing the cocci in 30 minutes as does the dimethyl). Gonococcus colonies develop a pink color which on further oxidation becomes maroon and finally black, Streptococcus and diphtheroid colonies fail to undergo this color change. Caution is indicated not to be mislead by a mere darkening of the surrounding media. Spreads made and stained from the oxydase-positive colonies must verify the tinctorial and morphological properties of the micro-organisms as this stain is not entirely specific for the Neisseria group. Medium sized, convex and translucent colonies which give the oxydase reaction may be accepted as gonococci if they consist of Gram-negative diplococci; in cases of doubt, i.e., if appearance of colonies is not entirely characteristic or when the complete identification is of special importance, subcultures are made and the fermentation reactions and ability to grow on ordinary agar are determined (The dye does not interfere with the staining properties of the gonococcus though it does interfere with its cultivation if it has proceeded beyond the pink stage).

NEISSERIA INTRACELLULARIS

(Meningococcus)

Characteristics: Similar to the gonococcus, but found in different locations and possessed with different invasiveness; distinguishable by serological tests. Divided into five types by serological behavior, types I and II and less commonly types III, IV and V; types I and III, and II and IV, respectively, are very closely related. Responsible for endemic and epidemic cerebrospinal meningitis in man; may be found in and isolated from infected spinal fluid, blood or nasopharyngeal secretions of patients suffering with cerebrospinal meningitis and from the nasopharyneal secretions of carriers. Highly susceptible to inimical agencies; cocci die in less than 3 hours when dried and kept at room temperature; killed by moist heat at 55° C. in less than five minutes; cultures die in a few days when kept at room temperature.

Identification:

1. Macroscopic appearance of the spinal fluid is to be noted and reported. Normal fluid is water-clear and colorless. Meningitis fluid is more or less turbid. Color, turbidity, blood and clot are to be noted. Blood, if fresh, may have come from the spinal puncture and make examination of the fluid difficult.

2. Microscopic: An immediate presumptive diagnosis of meningococcic meningitis may be made by direct study of cerebrospinal fluid.

a. Stained films of suspected final fluid: centrifuge the fluid, prepare spreads of the sediment on glass slides, fix and stain by Gram's method. Examine for typical Gram-negative, coffee-bean shaped, intracellular diplococci. If present, they should be considered as meningococci and tentatively reported as such, to be confirmed by culture and agglutination tests. The presence of other organisms and the relative number and kind of tissue cells are also reported.

b. Cell counts of spinal fluid: make total and differential counts, comparable to the counting of blood cells. The relative number of polymorphonuclear and mononuclear leucocytes are to be noted - the former are usually enormously increased in cerebrospinal meningitis.

3. Culture of Sediment of Spinal Fluid.

a. Plant several loopfuls of sediment on warm blood agar plate.

b. Inoculate tube of warm serum dextrose broth with 1 cc.

c. Incubate cultures at 37°C. for 18-24 hours and observe for the typical Gram-negative, coffee-bean shaped diplococci. Cultures are generally pure; if mixed, pure growth is to be obtained by subcultures on solid media (as for the gonococcus). Pure cultures are used for fermentation tests to rule out N. gonorrhoeae, and for tube-agglutination tests.

4. Culture of blood: This is not a routine procedure; the meningococcus may be recovered from the blood by routine methods, in anomalous infections with septicaemia, with or without meningitis.

5. Culture of nasopharynx: This is done for the detection of carriers only. The nasopharynx of convalescents and of potential carriers are touched with a sterile applicator or inoculating needle, and this inoculum is spread diffusely onto warm blood agar or chocolate agar plates; after incubation at 37° C., suspect colonies are fished to warm serum dextrose broth for confirmation of identity.

6. Agglutination tests of pure cultures: A presumptive slide agglutination may hasten the procedure and cast out atypical organisms. A macroscopic tube-agglutination test with polyvalent meningococcic antiserum is used for final proof of identity. Occasionally type determination will be indicated. Most of the saprophytic Neisseria are salt or serum sensitive; to rule out non-specific clumping it is necessary, in all agglutination tests for meningococci, to run controls using normal horse serum (diluted 1:10) and saline.

a. Presumptive test: Place a drop each of polyvalent antimeningococcic serum (1:10), normal horse serum (1:10) and sterile saline on separate areas of a slide; emulsify bacteria (portion of suspected colony) in each drop; observe for clumping of organisms.

b. Macroscopic test tube agglutination test: Add 0.5 cc. amounts of each sera, diluted to ½ of titer shown on vial, into labeled tubes; use separate tube for polyvalent and for each type antimeningococci serum (usually I & II only) to be tested; another tube receives 0.5 cc. of normal horse serum (diluted 1:10) and last tube receives 0.5 cc. saline; to each tube add 0.5 cc. of suspension of cocci being tested; incubate overnight at 45-55°C. or for 2 hours at 37°C. and overnight in icebox.

c. If the organism is a meningococcus, it should agglutinate in tube containing polyvalent and homologous type serum and not in other tubes. If clumping occurs in either control tube, the test is "unsatisfactory".

7. Fermentation reactions; with material from a pure culture, inoculate tubes of serum water media containing 4 pivotal sugars (see chart) and incubate at 37°C.

Neisseria catarrhalis

Characteristics: Gram-negative diplococci; in sputum, the organisms are shaped like coffee-beans and may be both intra and extracellular; in cultures they are generally larger and are found in pairs and tetrads; grow freely, forming large colonies in 24 hours. They are normally found in the nose and throat; have meager pathogenicity; may be found, incidentally, in inflammatory secretions especially of respiratory area. A number of closely related Neisseria, also found in respiratory area, are included on differentiation chart.

Identification:

1. Microscopic: Make gram-stained spreads of the infected secretions and examine for Gram-negative cocci. These organisms are larger than meningococci, may not be arranged in pairs, may be intracellular.
2. Culture:

a. Inoculate plain agar, incubate at 22° C.; N. catarrhalis will grow, gonococcus and meningococcus will not.

b. Pure culture is inoculated into sugar series in serum water media. (See chart for results)

Differentiation of Various Species of Neisseria.

	Dextrose	Maltose	Levulose	Sucrose	Agar Growth	22°C. Growth	Agg. with Meningococci serum	Special colony feature
N. gonorrhoeae	A	-	-	-	-	-	-	Small, round, convex.
N. intracellularis	A	A	-	-	[illegible]	-	+	Small, round, bluish-grey
N. catarrhalis	-	-	-	-	+	+	-	Large, greyish-white.
N. sicca	A	A	A	A	+	+	-	Large, wrinkled, impossible to emulsify.
N. perflava	A	A	A	A	+	+	-	Greenish yellow, adherent to medium.
N. flava	A	A	A	-	-	-	-	Yellow
N. subflava	A	A	-	-	+	+	-	Greenish-yellow, adherent to medium.
N. flavescens	-	-	-	-	?	?	-	Golden-yellow

A indicates formation of acid.

VIBRIO COMMA

Description: Slightly curved rods with rounded ends often resembling a comma; occur singly, in S-shaped pairs, short chains or spirals; actively motile; grow readily aerobically on simple media at 37°C.; agar plate colony: 1-2 mm. diameter, greyish yellow, translucent, low convex, with smooth or finely granular glistening surface and an entire edge, butyrous consistency; broth growth: abundant, with powdry deposit, thick surface pellicle.

Identifying Characteristics:
(a) Their power to grow on solid media which are so alkaline (pH 8.0 to 8.4) that other organisms cannot develop.
(b) Their initial growth at surface of liquid media, while accompanying organisms grow throughout the liquid.
(c) Cholera red reaction ≠ (also given by two saprophytic species)
(d) Indol ≠, M.R. -, V.P. -; acid, no gas in glucose, levulose, galactose, maltose, mannite, and sucrose; lactose may become acid after 14 days; litmus milk alkaline at top, slightly acid at bottom, not coagulated, slowly peptonized; nitrites produced from nitrates.
(e) Gelatin stab growth: good filiform growth, confluent at top, discrete below, funnel-shaped liquefaction, with thick yellowish-brown pellicle on surface.
(f) Agglutination with cholera immune serum.

Cholera-like Vibrios: There are several classified and probably many unclassified vibrios isolated from feces or water and differentiated on serological and biochemical characteristics.

Examination of Clinical Material: Vibrio comma may be isolated from the stools or intestinal contents of cases or carriers, from contaminated water or foods and identified by microscopic, cultural and serological methods.

Specimen Collection:
(a) The "rice water" stool of cases or the feces of carriers are transmitted without the addition of glycerol or other preservative.
(b) Surface water transmitted in a sterile liter flask.

Microscopic: A presumptive diagnosis of suspected cases, not of carriers, may be quickly made by examining stained spreads of flakes of mucus from the "rice water" stool; stain by Gram's method and with dilute-carbol-fuchsin; if Gram negative, comma-shaped organisms are present, examine a hanging drop preparation. Presumptive positive report may be made if large numbers of typical, actively motile, vibrios are found. This finding must then be confirmed by cultural and serological examination.

Cultural: Specimens of feces from suspected cases or carriers should be planted, using two or more loopfulls of intestinal mucus or liquid feces, with the least possible delay and incubated at 37°C.
(a) Alkaline peptone water pH 8-8.4 (several tubes)
(b) Alkaline nutrient agar pH 8-8.4
(c) Dieudonne's agar.

Water under test is placed in 100 cc. amounts in sterile flasks, to each flask is added 10 cc. of 10% peptone water. After 6-12 hours incubation at 37°C., transfer a portion of the surface growth to the three media above.

Rapid Presumptive Test

After 6-8 hours at 37°C, examine hanging drop and stained film preparations made from the surface growth of peptone water. If Gram-negative, motile vibrios in large numbers are noted, test as follows:
(a) Microscopic Agglutination Test: Deposit near one end of a slide a drop of agglutinating serum of a dilution of 1:200 (titre not less than 1:4000) and near the other end a drop of saline; also place a third drop consisting of normal serum (diluted 1:10) near center of the slide as a control; then touch the suspected surface growth with point of the inoculating needle, rub up in the drop of saline solution; flame the point, again touch the surface pellicle with the point and rub it in the drop of serum dilution; flame the point of the platinum needle again and add bacteria to the serum control in the same manner, agglutination will almost instantly appear in the anticholera serum (if cholera); the drops may be allowed to dry, then fix and stain; if agglutination has taken place, it will be evident in the stained specimen to the naked eye or on slight magnification with the hand lens.
(b) Cholera Red Test: A few drops of concentrated sulphuric acid are added to a 24 hour peptone water culture; a resulting red color depends upon the nitroso-indol reaction from the production of indol and the reduction of nitrite in the peptone.

Confirmatory Tests: If either of the presumptive tests are positive, obtain pure cultures for confirmation by selecting isolated colonies from plate media and transferring to:
(a) Gelatin tube: to note characteristic type of liquefaction.
(b) Alkaline peptone water: for cholera red test.
(c) Agar slant for macroscopic tube-agglutination test.

Pfeiffer's Phenomenon: Small loop full from colony in 1 cc sterile saline mix with 1 cc cholira immun serum 1:1000 dil. inject I.P. into G.P. after 20 min. withdraw peritoneal fluid c̄ cap pipettes--if pos. non motile disintigrating vibrios can be seen microscopically.

Pasteurella
(Hemorrhagic septicaemia group)

Characteristics: Small Gram-negative rods showing bipolar staining. Aerobe, facultative anaerobe. Non-motile or motile. Frequently pathogenic, producing characteristic hemorrhagic infections in man and animals. Includes:

P. pestis - causing plague in man and rodents.
P. tularensis - causing tularaemia in man and rodents.
P. avicida)
P. muricida)associated with fowl cholera or
P. cuniculicida)hemorrhagic septicaemia of birds or lower animals.

Pasteurella pestis

Habitat: A parasite of rats and other rodents, causes plague in man. Transmitted by the bite of infected rat flea, or by contact or contamination with rodent, or human case or carrier.

Characteristics: Short, thick bacillus; pleomorphic, especially in 3% salt agar; bipolar staining; grows readily on agar at 37°C. with raised, translucent, grayish-yellow, glistening, viscid growth.
May live for months in bodies of dead animals. Agglutinated by plague antiserum. Infectious by inoculation for small animals; subcutaneous injection into guinea pigs provokes local oedema followed by inflammatory swelling of regional lymph nodes, and a generalized infection to death in 2 - 5 days; postmortem appearence: glands enlarged, surrounded by hemorrhagic exudate; small grayish, necrotic areas in liver and spleen; bacilli found in local lesions, bubo, internal organs, especially spleen, and blood.

Collection of Specimens:
1. Pus or gland fluid from bubos,aspirated by syringe or collected after incision (may be forwarded to distant laboratories on agar slants).
2. Portions of affected tissues, removed at operation, to be forwarded in sterile bottles.
3. Blood specimens, taken during period of septicemia.
4. Autopsy materials, preferably bubo, lung, liver and spleen.
5. Sputum, in cases of pneumonic plague.
6. Rodent: The whole rodent, shipped in fruit preserving jar, sealed.

Microscopic Examination:
1. Stain films from suspect materials by Gram's method and methylene blue or dilute carbol-fuchsin (for bipolar staining).
2. The presence of typical Gram-negative, short, ovoid, polar-staining bacilli, including many degenerated and poorly stained forms, is suggestive but not conclusive evidence of P. pestis infection.

Culture:
1. Inoculate surface of blood agar, glycerol agar and 3% NaCl agar plates.
2. Plant blood specimen into nutrient broth and incubate before plating.
3. Incubate cultures at 30 to 35°C. for 36 to 48 hours.
4. Observe growth and transfer to agar, broth, litmus milk, gelatin, tryptone broth, lead acetate medium and sorbitol broth for further study. (See chapter "Classification of Bacteria").

Agglutination: (Macroscopic method preferred, to avoid the spontaneous clumping confusing the microscopic test)
1. Make suspension of young agar culture in normal saline, using only the fine supernatant emulsion remaining after period of settling.
2. High-titre agglutinating serum (horse) is generally used.
3. Test is of greatest value in identifying suspect cultures, positive titre being interpreted in comparison with the titre of same serum tested with a known plague antigen.
4. Test is of little value as applied to patient's serum, for agglutinins do not appear in patients suffering from plague until about 9th day.
5. Salt solution controls are necessary in all tests to detect auto-agglutination.

Animal Inoculation:
1. Caution: Animals should be freed of all ecto-parasites, prior to use, by dipping in an antiseptic solution. Then place in glass jars covered with fine mesh gauze to prevent access or escape of any parasites. When handling animals, living or dead, protect the hands and arms by wearing rubber gloves and long sleeved gown.
2. Inoculate guinea pigs or mice subcutaneously with small amount of the original specimen or with a loopful of suspected culture. Putrefied materials may be applied to the freshly shaved abdomen of a guinea pig (plague bacilli penetrate the abraded skin, contaminants do not).
3. If P. pestis is present, the animals will develop characteristic lesions, die in 2 - 5 days with characteristic postmortem appearance; cultures of P. pestis may be isolated from the lesions.

Diagnosis of Plague in Rodents:
1. Postmortem appearance will usually evidence the natural infection in rodents.
   a. Bubo, with hemorrhagic spots and areas of gray necrosis.
   b. Subcutaneous and general congestion.
   c. Granular liver, with punctate hemorrhage and grey-yellow spots.
   d. Congested spleen.
   e. Pleural effusion.
2. Bacilli may be found in bubo, liver, spleen and blood, and isolated from thence for study in pure culture by methods used for clinical materials.
3. Shipment to a distant laboratory for examination: The entire carcass is placed, without any preservative, in a tightly sealed container, which is packed in a second container to avoid breakage and escape of contents. The package must be shipped by express; federal laws prohibit the shipment of plague-infected materials by mail. Decomposition may be avoided by surrounding the inner container with ice or "dry ice". Label package "Perishable - for Bacteriological Examination - Please Expedite."

Pasteurella tularensis

Characteristics: Small, Gram-negative, non-motile rods; pleomorphic, bacillary and coccoid forms; stained best with carbol-fuchsin and crystal violet, show bipolar staining; fail to grow on ordinary media; aerobic; require specially enriched media for growth; an organism which grows on plain agar

or in broth is not P. tularensis; growth on serum-glucose-cystine agar, 2 to 5 days at 37°C.: minute, greyish-white colonies. Fairly susceptible to inimical agencies; killed by moist heat at 56 C. in 10 minutes. Agglutination tests of great value in diagnosis of disease by serum study, or in identification of cultures; agglutinins may persist for 20 years after recovery and a positive serum agglutination does not necessarily mean active infection. P. tularensis antiserum also agglutinates Brucella antigens to about ¼ of its titre. P. tularensis is the cause of "tularemia", a plague-like infection of rodents, especially rabbits, and occasionally in man. Generally transmitted from rodents to man by infected blood-sucking insects, such as flies, ticks, lice, fleas and bedbug, or by direct handling of infected rabbits or squirrels. Accidental laboratory infections occur due to its ability to invade unbroken skin.

Microscopic Examination:
Of value (1) to study morphology of organisms and (2) to rule out M. tuberculosis by observing acid-fast stain of spreads made from pathological materials.

Culture:
1. Piece of infected tissue, pus, fluid or blood is planted on slants of glucose-cystine agar or blood cystine agar. Incubate at 37°C. for 3 to 5 days.
2. Blood agar plates also are planted to detect other organisms.
3. Observe cystine slants for characteristic colonies. If negative, continue observation for 21 days; if growth occurs, identify organism by stained spread, pure culture transplants and macroscopic agglutination tests with high titre immune serum.
4. Cultures made from blood and lesions of man are usually unsatisfactory. Cultures should be made from heart's blood, spleen, lymph nodes and liver of guinea pigs following inoculation with material from patient.

Animal Inoculation:
1. Inoculate guinea pigs, rabbits, or mice with suspected materials from glands, ulcers or blood: (a) subcutaneously and (b) rubbed on the recently shaven, abraded abdomen if other bacteria are present.
2. Result: Death in 5 to 10 days (generally) with characteristic lesions:
   a. At site of inoculation, hemorrhagic oedema, no pus.
   b. Bubos, cervical, axillary or inguinal.
   c. Glands enlarged and filled with dry, yellow, caseous material.
   d. Spleen enlarged dark.
   e. Liver contains discrete, white caseous granules.
   f. Organisms can be seen in spreads and be cultured from spleen, liver bubo and blood.

Agglutination Reaction: Macroscopic tube method preferred.
1. Set up agglutination tests of patient's serum against P. tularensis and Brucella (abortus or melitensis) antigens. Incubate in water bath at 45 - 55°C. for 12 to 18 hours.
2. Agglutination of P. tularensis by serum in dilutions of 1 to 80 or higher is considered diagnostic of tularemia, provided there is no cross agglutination with Brucella. Agglutinins appear in the patient's blood after the first week of the disease and usually increase rapidly.
3. Identity of a suspect culture may be established by a similar test using a suspension of the organisms and serial dilutions of a P. tularensis antiserum of known titre. The resultant agglutination to be significant, must be present in dilutions approaching the known titre of the serum.

Genus Brucella

Description: Minute rods with many coccid cells; 0.5 by 0.5 to 2.0 microns; Gram-negative; do not show bipolar staining; all species pathogenic to man are non-motile; do not liquefy gelatin; and fail to ferment any carbohydrates.

Habitat: Strict parasites, invading animal tissue, producing infection of the genital tract, the mammary gland or the lymphatic tissues and the intestinal tract. Br. melitensis, Br. abortus and Br. suis, primarily infect goats, cows and hogs, respectively, causing abortion and systemic infection; infectious to other domesticated animals; may infect man causing undulant fever (brucellosis). The motile species, Br. bronchiseptica causes distemper in dogs; also causes acute infection in other animals; and rarely infects man.

Br. melitensis, Br. abortus and Br. suis.

Description: Gram-negative, non-motile coccobacilli as for genus: Br. melitensis and Br. suis grow aerobically, Br. abortus requires 10% CO_2 for initial isolation and early culture transplants; growth on all media is slow, grows best on glucose liver infusion agar with pH 6.6: 48 hour colonies on plate are small, circular, convex, amorphous, smooth, glistening and entire; agar cultures turn media brownish after 7 days. The three species are very closely related; may be separated with difficulty on basis of (1) CO_2 requirement for growth, (2) growth on media containing certain dyes, (3) H_2S production and (4) agglutinin absorption tests.

Habitat: Found in blood, urine, feces, exudates and occasionally sputum and nasal drainage of human cases; also in milk, cheese and other dairy products from unpasteurized milk from infected animals.

Laboratory examination of clinical material:
1. Microscopic.- Indistinguishable morphologically. However, Gram-stained smears from pathological lesions should be examined for the small Gram-negative rods described above.
2. Cultural.-While the organisms may be found in the blood early in the disease and during febrile periods and in urine and milk specimens at irregular intervals, the percentage of positive cultures, even from proved cases, is low.

a. Obtain specimen consisting of 10-12 cc. of blood or 50 cc. of urine or milk. Other body foci such as contents of ovarian cyst, synovial fluid, or excised glands may also be subjected to cultural study.

b. Inoculate two flasks containing 100 cc. of veal infusion broth pH 6.6 with 5 cc. of blood, several loopfulls of sediment from catheterized urine specimen, or several loopfulls of sediment and of cream layer from milk. Also streak specimen on two infusion agar plates.

c. Incubate one set of media in incubator 37°C. for growth of Br. melitensis and Br. suis; place other set of media in jar containing 10% CO_2 and incubate at 37°C. for Br. abortus.

d. Examine plates and Gram-stained films from broth after 24-48 hours and at frequent intervals thereafter for growth. Streak new plates from broth at least once per week, even if no evidence of growth is discernible. Observe cultures for at least 4 weeks before reporting as negative.

e. Identify any positive culture as belonging to this group by agglutination with antisera prepared against either Br. abortus, Br. melitensis or Br. suis.

Note: Although not usually required the species of young cultures can be determine by (1) agglutinin absorption tests, (2) tests for H_2S production, and (3) ability of the organism to grow on media containing certain dyes (basic fuchsin and thionin).

Table. - Differential characters of the three related species of Genus Brucella.

	10% CO_2 required for Primary isolation	H_2S formation (days).	Growth on media containing: Thionin	Basic fuchsin
Br. melitensis	0	<1	+++	+++
Br. abortus	++	2	0	+++
Br. suis	0	4	+++	0

3. Animal inoculations. - Br. melitensis and Br. suis, and less constantly Br. abortus, may be isolated from infected material by subcutaneous inoculation into guinea pigs (preferably males); after 4 weeks, kill the animal; examine Gram-stained smears from the lymph glands, spleen and liver; and make cultures from the liver, spleen, blood and lymph nodes. This test is seldom used because of the great danger of laboratory infection.

4. Serological. -

a. Identification of pure cultures:

There is complete cross agglutination to titer between an antigen prepared with either species and antisera prepared against any other species. However, Br. abortus and Br. suis can be differentiated from Br. melitensis, but not from each other, by agglutinin absorption tests.

b. Serum from a patient taken after the fifth day of disease will usually contain agglutins. Set up macroscopic agglutination tests in dilution of 1/20 to 1/640 or higher against a Brucella antigen (abortus, melitensis or suis) and against Pasteurella tularensis antigen. Agglutination of Brucella antigen in dilution of 1/100 or higher is considered to be significant. Cross agglutination in serum from patients with Brucellosis or tularemia is frequently present, but is less marked with the heterologous antigen. Agglutinins may persist for years after recovery. This is the most valuable test for diagnosing Brucella infections and is the only one routinely used.

Genus Hemophilus

Characteristics: Minute rods, sometimes almost coccoid, sometimes thread-like and pleomorphic; Gram-negative; not acid-fast; non-motile, non-sporing, non-encapsulated. Strict parasites, do not grow on common media, require for their cultivation accessory substances present in the blood and fresh vegetable tissue. H. influenzae, H. ducreyi and H. pertussis are the three most important species.

Hemophilus influenzae

Characteristics: Very small, short rod; stains faintly, best by dilute carbol-fuchsin or Giemsa stain; grows best on media containing hemoglobin, subcultures on plain or serum agar fail to grow; chocolate agar plate colony, 24 hours at 37°C.: small, pinpoint, transparent, smooth, raised; tendency to grow best near colony of other aerobic organism, i.e., "Satellite" colonies; not subject to agglutination test; does not have conspicuous biochemical activities.

Habitat: Commonly found in cultures of upper respiratory tract, their significance there questionable; probably not as name implies, related to the disease influenza. Occasionally found in pathological spinal fluids. "Koch-Weeks" bacillus, formerly called H. conjunctivitidis, found in eye fluids in acute infectious conjunctivitis ("Pink eye"), is now classified as H. influenzae.

Identification:

1. Koch-Weeks bacillus:
   (a) Make slide spread from conjunctiva, stain by Gram's method and with dilute carbol-fuchsin.
   (b) Observe for minute Gram-negative bacilli; often intracellular.
   (c) Culture is not informative except to reveal other organisms.
2. Spinal fluid, respiratory tract and other suspect materials:
   (a) Make culture on chocolate agar, incubate at 37°C. for 2 days.
   (b) Suspect colonies are identified on colony appearance, microscopic morphology of organisms and failure of subcultures to grow on plain agar. Colony may be confused with a streptococcus colony.
   (c) Specific identification considers source of the specimen, hemolytic properties and requirements of accessory growth factors.

Hemophilus pertussis

Characteristics: Like H. influenzae except bacilli are more uniform in size, with less pleomorphism, ferment no carbohydrates and do not require accessory factors for growth, but cannot be distinguished on morphology alone; colony on potato-glycerin-blood medium (pH 5.0) at 37°C. barely visible in 24 hours, plainly visible after 48-72 hours as small, greyish, raised, pearl-like growth; after several generations growth is freer, glistening, becoming in a few days heavy, almost like the growth of typhoid bacilli; then transplants will grow on plain agar.

Habitat: Constantly present in the respiratory secretions of whooping cough.

Identification: Cough plate method for isolating *H. pertussis* is preferable to sputum cultures: Open Petri dish, containing potato-glycerin-blood medium, is held in front of the mouth during a cough paroxysm. The organisms, sprayed on the plate with droplets of secretion appear in colonies after 37°C. for 48 hours. Colonies are larger, more opaque and whiter than those of H. influenza.

Hemophilus duplex

(Morax-Axenfeld bacillus)

Characteristics: Short stumpy, moderate size bacillus, often in diploform and chains. Cultivated only on media containing blood, serum or ascitic fluid. On Loeffler's blood slant colonies appear after 24-36 hours at 37°C. as small identations which indicate a liquefaction of the medium.

Habitat: Found in eye in subacute infectious conjunctivitis. Not pathogenic for animals.

Identification:
1. Prepare slide spreads from conjunctival sac, stain with dilute carbolfuchsin or Gram stain.
2. Short, stumpy bacilli in direct spreads are presumptively Morax-Axenfeld bacilli.
3. Culture on Loeffler's blood slant or other special media may confirm.

Hemophilus ducreyi

Characteristics: Very small ovoid rod, non-motile, tendency to be in short chains and parallel rows; Gram-negative; tendency to be more deeply stained at the poles. In pus, the bacilli are often found within leucocytes. Difficult to cultivate; coagulated blood which has been kept for several days in sterile tubes (fresh blood will not do unless heated to 55°C for 15 minutes) has been found to be a favorable medium.

Habitat: The cause of chancroid, the soft chancre, are found in the pus of ulcerating chancroidal ulcers, mixed with secondary infection, and in purer state in the chancroidal bubo. Not inoculable to lower animals.

Identification:
1. Examination of spreads or cultures for *H. ducreyi* is seldom practiced because of the technical difficulties of identification and the fact that chancroid lesions are usually distinguishable as such without laboratory confirmation.
2. Direct diagnostic cultivation from chancroidal lesion:
   (a) Media: 1 cc. of sterile rabbit blood (freshly drawn) is placed in each of several small tubes, allowed to clot, then heated to 55°C. for 15 minutes and kept in icebox until used.
   (b) Thoroughly cleanse lesion with sterile water or salt solution.
   (c) Scrape material from bottom of ulcer or from beneath its edges, with a stiff platinum loop and plant in a tube of clotted blood by passing the wire around the clot.

(d) After $37^{\circ}C$. for 24 hours, the serum around the clot is stirred with the platinum loop and a spread is made and examined by Gram method.

(e) Characteristic chains of Gram-negative bacilli, sometimes in pure, sometimes in mixed culture, will sufficiently identify the organism.

(f) Transfer onto soft moist blood agar of pH 7.2 may give in 48 hours pinhead size, transparent, grey colonies with a firm, finely granular consistency.

3. Culture from unruptured bubo; pus is withdrawn by aspiration with a sterile hypodermic syringe and needle; cultured as above.

GRAM-NEGATIVE, AEROBIC, NON-SPORE-FORMING ENTERIC BACILLI
(FAMILY ENTEROBACTERIACEAE)

Characteristics: Gram-negative rods, widely distributed in nature. Grow aerobically. Many species are parasitic for man, several of which cause typical disease; other species are saprophytes, or parasites on plants and animals. Grow well on ordinary culture media. All species attack certain carbohydrates forming acid, or acid and visible gas. May be motile or non-motile. Non-spore forming. Has been divided into five tribes, only three of which (Eschericheae, Proteae and Salmonelleae) contain species of interest in Medical Bacteriology. All of these bacteria are morphologically similar. They have many other characteristics in common, and serological as well as cultural methods may be required to definitely identify a member of the group.

THE COLI-AEROGENES GROUP
(Tribe Eschericheae)

Characteristics: Motile or non-motile rods, commonly occurring in the intestinal canal of normal animals, in the respiratory tract of man, or widely distributed in nature. All ferment dextrose and lactose with the formation of acid and visible gas. Do not liquefy gelatin except slowly by one species (Aerbacter cloacae). Separated into three genera on basis of results of methyl red test, Voges-Proskauer test, and ability to utilize citric acid as sole source of carbon. See table below.

Genus and species	Methyl red test	Voges-Proskauer test	Indol test	Citrate utilization	Gelatin liquefaction	H_2S formed
Escherichia coli	+	-	+	-	-	-
E. freundii	+	-	(+)	+	-	+
Aerobacter aerogenes	-	+	(-)	+	-	(-)
A. cloacae	-	+	-	+	+	(-)
Klebsiella pneumoniae	(+)	(-)	-	(+)	-	-

Note: Some species give variable results; (+) or (-) indicates usual reaction.

* Page 570 Simmons Manual.

Escherichia Coli

Characteristics: Coccoid to long rods, occurring singly, in pairs and long chains. Gram-negative. Motile or non-motile. Not usually capsulated. Ferments many carbohydrates, including dextrose and lactose, with formation of acid and gas. The large number of species formerly identified on basis of motility and carbohydrate fermentation are now included within this species as varieties.

Habitat: Occurs in normal intestinal tract of animals; frequently found in soil and water, as a result of fecal contamination. Sometimes acquires pathogenic power and may cause local or general infections: frequently causes infections of the genito-urinary tract; invades the circulation in agonal stages of diseases.

Identification: 1. For isolating E. coli from water and sewage see section on "Bacteriological Examination of Water."

2. For E. coli in feces, urine, etc., follow the procedure outlined under examination of feces for E. typhosa and identify according to the reactions in chart above.

Aerbacter aerogenes

Characteristics: Short rods with rounded ends, usually shorter and plumper than E. coli. They are aerobic, Gram-negative, non-spore-forming and frequently capsulated. Ferment many carbohydrates, including dextrose, lactose and glycerol, with formation of acid and gas. Do not liquefy gelatin. Colonies on solid media are large and very viscid.

Habitat: Widely distributed in nature; normally found on grains and plants, sometimes found in the intestinal canal of man and animals. It has been reported as the cause of cystitis.

Identification: 1. See section on "Bacteriological Examination of Water" for method of isolating A. aerogenes from water.

2. Isolate organism from feces, food, and soil by plating on eosin methylene blue agar or other media as described under E. typhosa and identify by characteristic biochemical reactions shown in above table.

Klebsiella pneumonia

Characteristics: Short, plump, non-motile, Gram-negative rods; aerobic, growing well on ordinary media; produces a large, mucoid colony on solid media. It has a large capsule which can be demonstrated readily in spreads from sputum, animal exudates and other pathological material. Ferments dextrose, levulose, galactose, saccharose and usually lactose with production of acid and gas.

Habitat: Common commensal in respiratory tract; occasionally found in soil, dust and water. Associated with pneumonia and other inflammations of the respiratory tract. Occasionally found in various suppurative lesions of the body, and may give rise to septicemia.

Identification: 1. Examine stained spreads from pus, sputum, or fluid from lesions for Gram-negative encapsulated bacilli.

2. Inoculate eosin methylene blue agar plates or other media. Examine for mucoid colonies consisting of bacilli with typical morphology. Identify suspected colonies through cultural and biochemical tests.

3. Blood culture may be made by usual methods; identify any suspect colonies as above.

Genus Proteus.

Characteristics: (The only genus in tribe Proteae.) pleomorphic, Gram-negative rods; filamentous and curved rods, and involution forms are common. Generally actively motile. Characteristically produce ameboid colonies on moist media and decompose proteins; gelatin is rapidly liquefied by most species. Ferment dextrose and generally sucrose, but not lactose, with formation of acid and small amount of gas. Usually Voges-Proskauer test is negative. Genus consists of 8 species; type species is Proteus vulgaris.

Habitat: Putrefying animal and vegetable materials; found in feces, soil and gunshot wounds. Certain Proteus strains, identified as X19, X2 and X Kingsbury, originally isolated from typhus fever cases, are used as antigens in the Weil-Felix test (see section on Rickettsiae). One species, P. morgani, has been reported as the cause of mild enteritis.

Identification: 1. Most laboratories roughly identify any Gram-negative, motile bacillus that produces an ameboid colony on moist agar at 37°C. as belonging to the Proteus group and do not classify further.

2. However, one species of the genus, Proteus morgani, produces the typical ameboid colony only when grown on 1% agar at 21-28°C. Isolate pure cultures of this organism as described under E. typhosa, and identify on basis of fermentation of dextrose and other hexoses only, with formation of acid and slight amount of gas.

TYPHOID-DYSENTERY AND PARATYPHOID-ENTERITIS GROUPS. (TRIBE SALMONELLEAE)

Characteristics: Motile or non-motile, Gram-negative rods; grow aerobically; non-spore-forming; Voges-Proskauer test negative; gelatin not liquefied; and no spreading growth. Attack many carbohydrates with formation of acid, or acid and gas. Certain species of genus Shigella and genus Eberthella attack lactose with gas formation only. Tribe consists of three genera; genus Salmonella organisms ferment dextrose with the formation of acid and usually gas; genus Eberthella and genus Shigella organisms ferment dextrose with formation of acid, but no gas, Eberthella being motile and Shigella non-motile.

GENUS SALMONELLA

The organisms of this genus are defined as: usually motile, but non-motile forms occur. Attack numerous carbohydrates with the formation of acid and usually gas; lactose, saccharose and salicin are never attacked. Do not form indol or liquefy gelatin. Differ from coli-aerogenes group in failing to ferment lactose; and from typhoid-dysentery group in forming gas from dextrose. Can be separated into 37 species, several of which are pathogenic for man, causing a typhoid-like fever, food poisoning, or an acute gastro-enteritis. All species pathogenic for man are motile.

Important Species: 1. S. paratyphi, the cause of paratyphoid A fever in man. Characteristic reactions: never ferments xylose, rarely able to produce H_2S, and fails to utilize citrate and d-tartrate.

2. S. schottmuelleri, the cause of paratyphoid B fever in man. Characteristic reactions: ferments xylose and usually attacks inositol; H_2S formed; citrate +; and tartrate usually -.

3. S. typhimurium, a natural pathogen of rodents, especially mice, and many other animals; causes food poisoning in man. Characteristic reactions: very diffucult to distinguish from S. schottmuelleri, by means of either biochemical or serological reactions; most reliable tests for separating them being, (a) S. typhimurium is usually tartrate +, and (b) agglutination reactions with "H" antigens of organisms in the specific phase.

4. S. enteritidis, and its varieties, are widely distributed among animals; sometimes the cause of food poisoning in man. Characteristic reactions: ferments xylose, but never attacks inositol; H_2S +; citrate + and tartrate +.

5. S. hirschfeldii, the cause of a typhoid-like fever in man, sometimes referred to as paratyphoid C. bacillus; found principally in Europe. Characteristic reactions: biochemical reactions similar to those of S. enteritidis; serologically, closely related to S. choleraesuis.

6. S. choleraesuis, two varieties, causing American and European hog cholera, respectively; occasionally infect man. Characteristic reaction: fails to ferment arabinose, a carbohydrate attacked by other Salmonella.

Identification: 1. Isolate paratyphoid fever group from feces, urine or blood as described under E. typhosa.

2. Food poisoning group. Isolate pure cultures from feces or food. (See section on Food Poisoning).

3. Identify pure cultures by means of carbohydrate fermentations and other biochemical tests (see section on Classification of Bacteria) and by agglutination reactions.

4. The Salmonella group, including E. typhosa is very complex, serologically. Each species possesses from one to three distinct antigenic components in the body of the bacillus ("O" antigens) and other distinct components in the flagella ("H" antigens), the latter occurring in many species in two alternate phases, the specific phase and the group phase, each possessing different antigens. The same antigenic components may be found in several different species, in various combinations. However, most strains of the pathogenic species listed above can be definitely classified on basis of (a) source of specimen, (b) biochemical reactions and (c) series of agglutination tests.

GENUS EBERTHELLA

The organisms of this genus are defined as Gram-negative, motile rods, generally occurring in the intestinal canal of man, usually in different forms of enteric inflammation. Attack dextrose and several other carbohydrates with the formation of acid, but no gas; certain non-pathogenic species may attack lactose, saccharose and/or salicin with formation of acid, but no gas. E. typhosa is the only species regularly pathogenic for man.

Eberthella typhosa.

Characteristics: Actively motile, Gram-negative rods, possessing the general features of the tribe and genus. Never attack lactose, saccharose or salicin. The normal smooth motile form has one somatic and one flagellar antigen, thus producing both H and O agglutinins; non-motile variants are rare; the somatic antigens are related to those of Salmonella enteritidis and a number of other species of Salmonella. Colonies on plain agar, after 24 hours incubation at 37°C., are smooth, round, domed, greyish in color, transparent to opaque, with entire edge; after cultivation on artificial media, rough type variants may develop. See table in section on "Classification of Bacteria" for biochemical characteristics and table in this section for reaction on Russell's double sugar tubes, and for type colonies on differential plate media.

Habitat: Found in feces and blood, and occasionally in bile and urine, of patients ill with typhoid fever of which it is the causative agent; also present in feces, urine and bile of carriers.

Laboratory Examination of Specimen

The Specimens to be examined will usually consist of blood, feces, urine or bile of suspected cases of typhoid or paratyphoid fever and of bile, feces and urine of carriers for cultural study; also, of serum from patients for agglutination (Widal) test.

1. Microscopic examination. - This is of no value.

2. Culture - a. Feces, urine, bile, etc.:

(1) Spread the material, suspended in broth or saline if solid feces, over the dry surface of eosin-methylene blue agar, Leifson's dexoxycholate-citrate or other special differential media in Petri dishes, in such a manner as to insure the growth of well isoldated colonies. Also, inoculate specimen into tube of selinite broth or bile broth.

(2) Incubate 18-24 hours at 37°C.

(3) Study the plate cultures carefully, select several well isolated colonies of the type desired (see table) and from each inoculate Russell's double sugar (RDS) tube and plain agar slant.

(4) After 24 hours incubation examine cultures for type reaction on R.D.S. motility and Gram-staining properties.

(5) Identify any suspected pure culture (a) by inoculating various carbohydrate media and media for the other biochemical tests, and (b) by setting up macroscopic agglutination tests against known type antisera (E. typhosa, S. paratyphi, and S. schottmuelleri; other antisera may be used, if indicated).

(6) If at end of 24 hours plate cultures show no colonies of the type produced by pathogenic organisms, streak new set of plates from the broth culture and reincubate old plates for an additional 24 hours before discarding as negative.

b. Blood: Blood for culturing should be taken early in the disease, preferably during the first week.
(1) Obtain 10-15 cc. of citrated or defibrinated blood; whole blood can be used for immediate inoculation of media at bedside.

(2) Inoculate (a) flask containing 100 cc. of bile broth, 1% dextrose infusion broth or brilliant green broth with 2 to 5 cc. of blood (b) two agar pour plates with 1.0 cc. of blood each and (c) streak 2 or 3 loopfuls of blood on eosin methylene blue agar plate.

(3) Incubate at 37°C. and make daily transfers to blood agar and E.M.B. agar plates. If colonies develop, transfer to Russell's double sugar and identify by the procedure outlined above.

3. Serological examination. - a. A macroscopic tube-agglutination test, as indicated above, should be used to confirm the identity of an organism isolated from cultures; use suspension of suspected culture as antigen along with known type antisera.

b. Widal test: After the first or second week, demonstrable antibodies including agglutinins, develop in the blood of patients with an enteric fever. These may be demonstrated by Widal test. This test consists of a macroscopic (preferred) or microscopic agglutination test, using the patients' serum, and stock E. typhosa "H", E. typhosa "O", S. paratyphi and S. schottmuelleri antigens.

Genus Shigella

Characteristics: Small, Gram-negative, non-motile rods. Attack a number of carbohydrates with formation of acid but no gas.

Habitat: Several species are pathogenic for man, causing bacillary dysentery; other species may be found in the normal human intestinal tract; several species are pathogens of fowls and other small animals.

The Dysentery Group

1. Shigella dysenteriae (Shiga): A cause of dysentery in man and monkeys. Produces acid but no gas from dextrose, levulose and a few other carbohydrates. Never attacks mannitol, maltose, lactose or sucrose. Indol not formed. Serologically homogenous and different from the other species of Shigella.

2. Shigella sp. (Newcastle type): A cause of human dysentery. In peptone water solution, dextrose, maltose and occasionally dulcitol are fermented with acid production; lactose, mannitol and saccharose usually not fermented. Peculiarities of the organism are (1) occasionally a slight bubble of gas is produced from dextrose and dulcitol. (2) when dissolved in beef extract broth, dextrose, dulcitol and maltose are always fermented to acid and gas. Indol not formed. Serologically homogenous and not agglutinated by antisera prepared against S. dysenteriae or S. paradysenteriae.

3. Shigella paradysenteriae: A cause of dysentery in man, and of summer diarrhoea in children. Produces acid but no gas from dextrose and mannitol; some strains attack maltose or saccharose; dulcitol and lactose never fermented. Indol formation is variable. Has been divided into five races ("V", "W", "X", "Y" and "Z") by agglutination tests based upon the preponderance of one or another of four antigenic components, V, W, X and Z; considerable cross agglutination between races; seroligically distinct from S. dysenteriae and New castle's bacillus; slight cross agglutination with S. sonnei, S. alkalescens and S. madampensis.

4. Shigella alkalescens: Isolated from human feces and intestines; pathogenicity doubtful. Ferments dextrose, mannitol, maltose, dulcitol and sometimes saccharose; never attacks lactose. The most characteristic reaction is an initial and lasting, intense alkalinity produced in litmus milk. Serologically homogenous and distinct except minor cross agglutination with S. sonnei, S. paradysenteriae and S. madampensis.

5. Shigella sonnei: A cause of mild dysentery in man, or of summer diarrhoea in children. Ferments dextrose, mannitol, maltose, lactose, saccharose and several other carbohydrates with formation of acid, but no gas; dulcitol is never, and xylose seldom, attacked; fermentation of substances other than the monosaccharides may require days or weeks. Indol not formed. Serologically divisible into two types; some cross agglutination with S. paradysenteriae, S. alkalescens and S. madampensis.

6. Shigella madampensis (S. dispar): Isolated from human feces, apparently not pathogenic. Fermentation reactions similar to those of S. sonnei Indol is formed. Antigenically heterogeneous; may show slight cross agglutination with S. paradysenteriae, S. alkalescens and S. sonnei.

Laboratory Examination of Specimens

1. Microscopic examination: In bacillary dysentery, especially in infections with S. dysenteriae (Shiga), an early presumptive diagnosis can usually be made by direct microscopic examination of fresh fecal discharges.

a. Select portions of a very fresh specimen containing bits of mucus, bloody feces or shreds of the exudate. Prepare (1) thin films on slide and (2) cover slip preparations, both unstained and stained with Loeffler's methylene blue or 1% aqueous solution of brilliant cresyl blue, in order to study the cells present.

b. If the disease is the bacillary type of dysentery, microscopic examination will show blood in varying amounts, but usually abundant early in the disease; polymorphonuclear neutrophiles form about 90% of the exudate, and many of these show nuclear degeneration (ringing), while the cytoplasm frequently contains fat; endothelial macrophages, which are present in varying numbers are actively phagocytic and frequently contain engulfed bacteria, erythrocytes and leukocytes; these under go degernation and form "ghost cells"; plasma cells are present and are more abundant early in the disease; bacterial content is scanty.

c. For characteristic findings in amoebic dysentery stools, see section on Protozoology.

2. Cultural examination: Shigella may be isolated from the feces of patients and carriers by the methods indicated under Eberthella. However, both eosin methylene blue agar plates and desoxycholate-citrus agar plates should be inoculated, routinely, since the latter is an especially favorable culture medium for Shigella.

3. Serological examination: a. The suspected organisms may be identified by agglutination tests using polyvalent and species specific antisera; S. dysenteriae, S. paradysenteriae, S. sonnei and polyvalent (Shiga, Sonne and paradysentery) antidysenteric antisera are generally used.

b. Agglutination tests, using serum from patient against known antigens, are of limited value.

FAMILY ENTEROBACTERIACEAE

GENERAL CHARACTERISTIC: GRAM NEG., NONSPOREFORMING, AEROBIC, FOUND IN INTESTINAL TRACT, ALL FERMENT CERTAIN CARBOHYDRATES WITH FORMATION OF ACID AND SOME WITH GAS. MAY BE MOTILE OR NON MOTILE.

TRIBE PROTEAE (only one Genus-Proteus)

Gelatin liquified
Ferment dextrose and gen. Sucrose, but not Lactose, with formation Ac and small amt. gas.
Actively motile, amoeboid colonies on moist media.
Generally not pathogenic, one species P. morgani causes mild enteritis
Colonies colorless on E.M.B. Plates
Type Species - Proteus vulgaris

TRIBE ESCHERICHEAE

Gelatin not liquified (one exception)
All ferment Dextrose and Lactose with formation of Acid and Gas.
M. R. or V. P. positive
Motile or non motile
Generally not pathogenic to intestinal tract.
Colonies colored on E.M.B. Plates

Genus Escherecia

E. coli
- M.R. +
- V.P. -
- Indol +
- Citrate -
- Gelatin -
- H_2S -

E. fruendi
- M.R. +
- V.P. -
- Indol +
- Citrate +
- Gelatin -
- H_2S +

Genus Aerobacter

A. aerogenes
- M.R. -
- V.P. +
- Indol -
- Citrate +
- Gelatin -
- H_2S -

A. chloacae
- M.R. -
- V.P. +
- Indol -
- Citrate +
- Gelatin +
- H_2S -

Genus Klebsiella

K. pneumonia
- M.R. +
- V.P. -
- Indol -
- Citrate ±
- Gelatin -
- H_2S -

TRIBE SALMONELLEA

Gelatin not liquified
Lactose not fermented
Intestinal pathogen group.
Colonies colorless on E.M.B. Plates

Genus Salmonella
(Paratyphoid Fever)

Genus Eberthella
(Typhoid Fever)

Genus Shigella
(Dysentery)

TRIBE SALMONELLEAE

(Gen-Charact.-Gram. neg. rods non spore forming, Lactose not fermented, Gelatin not liquified)
V.P. -

- **Genus Salmonella** (Dextrose AG, Motility+, do not ferment Lactose, Sucrose and Salicin)
  - Maltose AG
    - Xylose AG
      - Arabinose AG
        - H_2S +
          - Inositol AG
            - S. schottmulleri
              - Citrate +
              - Tartrate ∓
            - S. typhimurium
              - Citrate +
              - Tartrate +
          - Inositol -
            - S. enteritidis & S. hirschfeldii
              - Citrate +
              - Tartrate +
        - H_2S -
          - S. typhisuis
          - S. Sp. Newcastle
      - Arabinose -
        - H_2S ±
        - S. cholerasuis
    - Xylose -
      - S. paratyphi
        - Indol -
        - Lit. Milk Sl Ac
        - H_2S ∓
        - AG from: Dextrose, Arabinose, Maltose, Dextrin, Mannitol, Dulcitol, Sorbitol
        - Citrate -
        - Tartrate -
  - Maltose -
    - S. pullorum
      - Indol -
      - Lit. Milk Ac → Alk
      - H_2S +
      - AG from: Dextrose, Arabinose, Xylose, Mannitol
- **Genus Eberthella** (Dextrose A no G, Motility +)
  - Sucrose -
    - H_2S +
      - E. typhosa
      - Ac from: Dextrose, Xylose, Maltose, Raffinose, Dextrin, Mannitol, Sorbitol
      - d-tartrate +
    - H_2S -, Rhamnose +
      - E. sp. Sendai
  - Sucrose Ac
    - E. oedematiens
      - Gelatin liquif.
      - Lactose Ac -
- **Genus Shigella** (Dextrose A no G, Motility neg.)
  - V.P. -
  - H_2S -
  - Mannitol -
    - Milk not coagulated
      - Indol -
        - Shig. dysenteria
        - Shig. sp. Newcastle
      - Indol +
        - Shig. ambigua
        - Shig. septicaemiae
    - Milk coagulated
      - Shig. minutissima
  - Mannitol Ac
    - Lactose -
      - Xylose -
        - Shig. paradysenteriae (Flexner)
          - Indol +
      - Xylose Ac
        - Dextrin -
          - Shig. alkalescens
            - Dulcitol Ac
            - Indol +
        - Dextrin Ac
          - Shig. gallinarum
    - Lactose Ac (slowly)
      - Dulcitol -, Sucrose Ac
        - Indol -
          - Shig. sonnei
            - Maltose +
        - Indol +
          - Shig. madampensis
      - Dulcitol Ac
        - Shig. ceylonensis

BACTERIOLOGY - CASE WORK SHEET

PATIENT ____________________ DATE ______________ SPECIMEN ______________

REC'D FROM ______________________________ ADDITIONAL DATA ______________

GRAM STAIN: MOTILITY:

	Hours			Days		Hours			Days
	24	48	72			24	48	72	
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

\# - doubtful ⊕ - acid & gas + - acid, no gas Alk - alkaline

REMARKS

(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ____________________ DATE ____________ SPECIMEN ____________

REC'D FROM ______________________________ ADDITIONAL DATA ____________

GRAM STAIN: MOTILITY:

	Hours 24	Hours 48	Hours 72	Days		Hours 24	Hours 48	Hours 72	Days
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful (+) - acid & gas + - acid, no gas Alk - alkaline

REMARKS
(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ______________ DATE ______________ SPECIMEN ______________

REC'D FROM ______________ ADDITIONAL DATA ______________

GRAM STAIN: MOTILITY:

	Hours			Days		Hours			Days
	24	48	72			24	48	72	
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful (+) - acid & gas + - acid, no gas Alk - alkaline

REMARKS
(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ______ DATE ______ SPECIMEN ______

REC'D FROM ______ ADDITIONAL DATA ______

GRAM STAIN: MOTILITY:

	Hours 24	Hours 48	Hours 72	Days		Hours 24	Hours 48	Hours 72	Days
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful ⊕ - acid & gas + - acid, no gas Alk - alkaline

REMARKS
(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ______________ DATE ______________ SPECIMEN ______________

REC'D FROM ______________ ADDITIONAL DATA ______________

GRAM STAIN: MOTILITY:

	Hours			Days		Hours			Days
	24	48	72			24	48	72	
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful ⊕ - acid & gas + - acid, no gas Alk - alkaline

REMARKS
(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ______________ DATE ______________ SPECIMEN ______________

REC'D FROM ______________ ADDITIONAL DATA ______________

GRAM STAIN: MOTILITY:

	Hours			Days		Hours			Days
	24	48	72			24	48	72	
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful ⨍ - acid & gas ⨍ - acid, no gas Alk - alkaline

REMARKS
(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ______ DATE ______ SPECIMEN ______

REC'D FROM ______ ADDITIONAL DATA ______

GRAM STAIN: MOTILITY:

	Hours 24	48	72	Days		Hours 24	48	72	Days
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful (+) - acid & gas + - acid, no gas Alk - alkaline

R E M A R K S
(If necessary, use other side)

BACTERIOLOGY - CASE WORK SHEET

PATIENT ______ DATE ______ SPECIMEN ______

REC'D FROM ______ ADDITIONAL DATA ______

GRAM STAIN: MOTILITY:

	Hours			Days		Hours			Days
	24	48	72			24	48	72	
R.D.S.					DULCITOL				
LACTOSE					SORBITOL				
MALTOSE					INOSITOL				
DEXTROSE					DEXTRIN				
SACCHROSE					SALICIN				
XYLOSE					INULIN				
RAFFINOSE					GELATIN				
ARABINOSE					d-TARTRATE				
RHAMNOSE					INDOL				
LITMUS MILK					V. P.				
H_2S PRODUCTION					M.R.				
SIMMONS CITRATE									
MANNITOL									

- doubtful (+) - acid & gas + - acid, no gas Alk - alkaline

REMARKS
(If necessary, use other side)

Characteristic Reactions of Gram-negative Intestinal Bacilli on Russell's Double Sugar Medium. - Phenol red Indicator (Alkaline is red, Acid is yellowish).

	Slant	Butt
Mechanism	The small amount of acid produced by dextrose (0.1%) is diffused, leaving alkaline slant. The larger amount of acid from lactose (1%) gives acid slant.	Organisms producing acid from either dextrose or lactose give acid butt. Salmonella give acid and gas (bubbles in medium). Typhoid-dysentery group produce acid only from dextrose.
Genus <u>Escherichia</u>	Acid	Acid and gas (++++).
Genus <u>Aerobacter</u> and Genus <u>Klebsiella</u>	Acid, returning to neutral or alkaline after several days.	Acid and gas (++++).
Genus <u>Salmonella</u>	Alkaline	Acid and gas (++).
<u>Shigella dysenteriae</u> and <u>Shigella paradysenteriae</u>	Alkaline	Acid
<u>Shigella sonnei</u> and <u>Shigella madampensis</u>	Alkaline. Small acid producing daughter colonies may be formed several days.	Acid
<u>Eberthella typhosa</u>	Alkaline	Acid
Genus <u>Proteus</u>	Alkaline	Acid and gas (+).

Colony Characteristics of Gram-negative Intestinal Bacilli on Differential Plate Media.

Medium	Eosin methylene blue agar	a. Desoxycholate agar. b. Desoxycholate-citrate agar.
Mechanism	Coli-aerogenes group ferment lactose and grow into large, opaque colonies; also absorb dye to give color to colony. The non-lactose-fermenting pathogenic species develop as small, colorless, translucent colonies	Non-lactose-fermenting organisms grow into small, clear, colorless, translucent colonies. On a lactose fermenting organisms produce large, reddish colonies. Growth on b is same except greater inhibition of coli-aerogenes group.
Genus Escherichia	Large colonies with large, dark, almost black centers, and with greenish metallic sheen.	a. Large opaque, reddish colonies; occasionally have colorless rim. b. Much inhibited; if present, growth is pink and opaque, opacity spreading to surrounding medium.
Genus *Aerobacter* and Genus *Klebsiella*	Large pinkish mucoid colonies with small, dark brown or black centers; rarely show metallic sheen.	a. Similar to Escherichia colony except larger and mucoid. b. Much inhibited; same as Escherichia shown in b above.
Genus *Salmonella*	Translucent, colorless or pinkish colonies, usually slightly larger than *E. typhosa*; later have bluish tint.	Large translucent colonies, domed, shiny, smooth and colorless.
Shigella dysenteriae and *Shigella paradysenteriae*	Small translucent, colorless colonies.	Same as Salmonella colonies except smaller.
Shigella sonnei and *S. madampensis*	Small, translucent, colorless colonies. Later may ferment lactose.	Same as *S. dysenteriae* colonies during first 24 hours, later may show reddish daughter colonies, or entire colony may become red.
Eberthella typhosa	Translucent, colorless colonies.	Translucent colonies; domed, shiny, smooth and colorless.
Alcaligenes faecalis	Translucent, colorless colonies.	Similar to *E. typhosa*
Genus *Proteus*	Translucent colorless colonies; slight spreading.	Same as Salmonella; spreading inhibited.

Cl.parabotulinum (Types A & B) and Cl.botulinum (Type C)

Habitat: These are primarily saprophytes of the soil, may occasionally be found in the intestinal tract of domesticated animals and on various foods contaminated by soil or dust. They are not infective to man or animals but do produce disease by means of the violent poison, toxin, it may produce in foods which act as culture media for its saprophytic growth. This toxin is not formed within the body. This poisonous toxin, variously applied, produces "botulism" or food poisoning in man, "forage poisoning" in animals or "limber neck" in poultry. The living organism may be sought for in the infected food but not in the poisoned man or animal for it is not an infection. Botulism may be associated with meat or meat products, fruits, vegetables, canned goods and various pickled and preserved foodstuffs. Broth cultures injected subcutaneously in mice, g.pigs, rabbits, cats, monkeys prove fatal in 1 to 4 days.

Morphology and Staining: Large sporulating rods with parallel sides and rounded ends, occurring singly or in chains. Slightly motile; not-capsulated. Gram-positive. Spores are oval, larger than the bacilli and usually situated at or near the end. Spores form best in sugar free media at a temperature of 20 to 25°.

Metabolism: Strict anaerobe growing well in ordinary media with neutral or slightly alkaline reaction. Optimum temperature 35° (growth poor at 37°). Hemolysis produced on erythrocytes (human and horse). Types A & B are generally proteolytic; type C only slightly proteolytic. Optimum pH 7.4 to 8. Above 37° toxin formation is impeded, below 20° toxin formation stops.

Cultivation:

Agar: 4 day growth: flat irregular, greyish yellow, filamentous colonies with alternately smooth and granular surface, and indefinite fringed periphery.

Deep glucose agar shake: 4 days growth: colonies thin semi-opaque discs with bi-convex brownish centers, translucent edges. Abundant gas formation.

Blood agar plate (Horse): 3 days: Irregular, round, 2-3 mm. colonies with smooth center fimbriate periphery. Alpha type hemolysis.

Cooked Meat Mediums (Brain): 4 days: Abundant growth, turbidity, gas formation, brain digested and blackened. Butyric acid odor.

Broth: 4 days: Dense turbidity, rancid odor.

Bio-chemical Reactions:

	"A"	"B"	"C"
Glucose	AG	AG	AG
Maltose	AG	AG	AG
Salicin	AG	AG -	-
Glycerol	AG +	AG +	AG -
Lactose	-	-	-
Inositol	-	-	AG
Indol	-	-	-
Nitrates	-	-	-
NH_3	+	+	+
H_2S	+	+	+
Meth.Red	-	-	-
V.P.	-	-	-
Litmus milk	Reduced and alkaline, no coagulation.		

Serology: Types A & B are identifiable by agg. and toxicity tests. Their toxin is specific only for type, the antitoxin of one is not neutralized by the toxin of another. Type C, forming another separate specific toxin is distinguished chiefly by lack of proteolytic powers.

Resistence: The bacilli without spores are readily killed by heat and chemicals. Spores withstand dry heat of 180° for 15 - 30 minutes, moist heat at 100° for 3 - 5 hours. Toxin is destroyed by 80° C in 5 - 15 minutes.

Identification: (see "Bacterial Food Poisoning")

CORYNEBACTERIUM DIPHTHERIAE
(Diphtheria bacillus)

Description: Slender rods, straight or slightly curved, of medium size; often lie at various angles to one another forming V or Y shapes, or clumped as Chinese letters; generally not uniform in thickness, exhibiting rounded, pointed or swollen ends or enlargements along the length of the cell; usually stain unevenly, showing barred and granular large forms, solid staining short forms; gram-positive, nonmotile; grow readily at 37°, preferably on Loeffler's serum or blood agar as small, circular, smooth, moist, grayish to creamy-white colonies, some strains giving narrow zone of haemolysis on blood agar; pathogenic to man and to guinea pigs.

Habitat: The cause of diphtheria, usually found in the mucous membranes of the nose, throat and larynx of cases and carriers, occasionally found as cause of conjunctivitis, wound infection, middle ear infection and broncho-pneumonia. It is not found in the blood stream, the general symptoms being caused by the powerful toxin formed at the local site of infection.

Identifying Characteristics: (1) Shape, size, irregular staining, V or Y arrangements as seen in a direct spread or spread from Loeffler's medium and stained by Loeffler's methylene blue or Neisser's stain.

(2) Growth freer on Loeffler's serum medium than that of other organisms.

(3) Colony form on blood agar. Colony on tellurite medium becomes black.

(4) Pathogenicity for guinea pig (see virulence test).

(5) Carbohydrate fermentation (see chart below).

Collection and Transmission of Specimen for Examination: Cotton swab may be applied to the involved area (throat, nose, wound) or to the membrane or exudate from that area with care to gather a considerable amount of the exudate on the swab, with caution not to contaminate the swab by it touching the tongue or other noninvolved areas. Use this swab for:

(a) Immediate inoculation of Loeffler's serum slant for 18-24 hours, incubation at 37°C., or for shipment to distant laboratory.

(b) Immediate inoculation of blood agar plate for incubation at 37°, 24 hours.

(c) Spread on slide for direct Neisser stain examination.

(d) (Optional) plant on "tellurite media".

Microscopic Examination: (Direct spread, Neisser's or Loeffler's stain). An immediate presumptive diagnosis can sometimes be made on the basis of morphology and staining features

of what few diphtheria bacilli may be observed in the direct smear, but here they will be confusedly mixed with the many other microorganisms of mouth or wound flora. Vincent's organisms and diphtheroids may give confusion, should be noted on report if found. Negative finding by direct method cannot be given value. Presumptive positive finding should be confirmed by cultural and virulence tests.

Cultural Examination: (a) Loeffler's tube, after 18-24 hours incubation at 37°C is examined by broad needle drag along its surface, this then spread on a slide and planted on blood agar and tellurite media for later pure colony isolation. The slide spread is stained by Neisser method and observed for diphtheria bacilli; the irregularity of staining and shape, the metáchromatic granules may be noted. If typical diphtheria bacilli are found and the culture is from a suspected case, a presumptive diagnosis should be made at once. If the culture is from a suspected carrier, diphtheria-like bacilli should be further identified by fermentation and virulence test.

(b) Blood agar plate will provide information on general bacterial flora, particularly streptococci, and will give opportunity for notation of colony form and single colony isolation of diphtheria-like bacilli.

(c) Tellurite media will point out the diphtheria-like colonies by black color.

(d) (Optional - rapid) Apply a sterile serum-swab to involved area, return to serum-tube and incubate for a few hours; transfer to other media and examine slide made by gently rolling swab out into thin film. Sterile serum swabs are prepared by placing sterile swabs into sterile tubes containing a few cc. of serum. Some such swabs are made to contain 2% potassium tellurite to attain blackening from growth of diphtheria bacilli.

Fermentation Reactions: C. diphtheriae can be differentiated from related organisms by their fermentation reaction in dextrose, saccharose and dextrin. The absence of power of a particular organism to ferment glucose and its ability to ferment saccharose is usually sufficient to exclude the organism from being a diphtheria bacillus.

Virulence Test: This is the only certain method by which the identity and virulence of C. diphtheriae can be confirmed or distinguished from nonvirulent varients. No other known species of this genus occurring in man produces a fatal toxaemia in guinea pigs. Pure cultures are preferred for this test, but for speedy test the suspension of a heavily positive Loeffler's tube may be substituted.

(a) Subcutaneous Method: Inject 2 cc. of a pure culture grown for 48 hours in infusion broth or 4 cc. of a Loeffler's slant suspension in 10 cc. of saline, subcutaneously into a 250 gram guinea pig. At the same time a similar injection of the culture is made into a control guinea pig which had been given 250 units of diphtheria antitoxin, intraperitoneally, 24 hours previously. If the

organism is a virulent diphtheria bacillus the unprotected animal will die within 3 to 5 days and on post mortem will show a gelatinous aedema around point of injection and enlarged hemorrhagic adrenals.

(b) Intracutaneous Method: Two guinea pigs of 250 grams are used, one of which has been injected intraperitoneally with 250 units of diphtheria antitoxin 24 hours previously. The growth from a 24 hour Loeffler's slant is suspended in 20 cc. of normal saline and .15 cc. is injected intracutaneously at corresponding site of each pig. Six cultures may be tested at the same time on two animals. Virulent strains of diphtheria bacilli produce a definitely circumscribed local infiltrated lesion which shows superficial necrosis in 2-3 days. In the control pig the skin remains normal. If a mixed culture was used for test, and contained streptococci or staphlococci with sufficient virulence, local lesions will occur in both animals; the test would then be considered inconclusive and repeated using a pure culture.

Schick Test: This is an intracutaneous skin test to evidence the presence or absence of immunity to C. diphtheriae. The injection consists of .1 cc. of diphtheria toxin (1/50 m.l.d.). A control test uses the same material which has been made inert by heat (75° for 5 minutes). Results are noted daily for 4 days and recorded as Positive, Negative, Positive Combined or Negative Combined reactions.

C. pseudodiphthericum (Hofmann's bacillus): This organism is shorter and thicker than C. diphtheriae, usually straight and clubbed at one end, rarely at both; when Loeffler stained it occasionally shows unstained transverse bands which, unlike these in C. diphtheriae, hardly ever exceed one or two. Sometimes the transverse band gives the bacillus a diplococcoid appearance; no polar bodies are demonstrable by Neisser's stain; it grows more luxuriantly, colonies larger, less transparent and whiter than are those of true diphtheria bacilli; a positive means of distinction is its inability to form acid on sugar media; it is not pathogenic to guinea pigs or to man. It is a common mouth commensal, may be found in 42% of normal throat cultures; diphtheria-like bacilli which prove to be avirulent generally are found to be C. pseudodiphthericum.

C. xerose (Xerosis bacillus): This is a harmless saprophyte, commonly found in the normal or inflamed conjunctiva of the eye. It closely resembles C. diphtheriae, and is indistinguishable morphologically and culturally though generally shorter; polar bodies may occasionally be seen; it differs in its acidifying action on sugar media and its nonpathogenicity to guinea pigs.

	Hiss Serum Water plus 1%			Virulence
	Dextrose	Saccharose	Dextrin	
C. diphtheriae	+	—	+	Virulent
C. pseudodiphtheriae	—	—	—	Nonvirulent
C. Xerose	+	+	—	Nonvirulent

Diphtheroid Bacilli: There is a large group of ill defined organisms given this general name because of their morphological resemblance to the diphtheria bacillus; often show metachromatic granules; are not virulent when tested by the guinea pig virulence test. They are common saprophytes of the throat, skin and other body areas, are so utiquitous that any association of them with specific disease must be avoided; they must be distinguished from virulent and therefore significant diphtheria bacilli.

MYCOBACTERIUM TUBERCULOSIS
(Tubercle bacillus)

Description: Slender rods, straight or slightly curved with rounded ends; occur singly, in threads or in clumps; may stain evenly or irregularly showing granular, beaded or banded forms; stain with difficulty but when once stained are acid fast; growth on media show, aerobic, aided by glycerine or other enrichments; growth on glycerine agar in 4 weeks at 37°C; colonies minute, crumb-like, irregular, whitish-yellow, later brownish, ridged, becoming dry; pathogenic to guinea pig.

Habitat: A strict parasite, causing tuberculosis of man, cattle and other animals; human and bovine varieties are distinguishable, both infectible to man; there are other species of this genus causing avian tuberculosis, infecting fish, snakes, turtles and other cold blooded animals. There are a number of acid fast bacilli which are strictly saprophytic but confusible with M. tuberculosis.

Identifying Characteristics:
(a) Acid fast bacillus, when stained by Ziehl-Neelsen method.
(b) Pathogenic to guinea pigs, producing tuberculosis in 6 weeks.
(c) Growth on special media slow, wrinkly and contains acid fast bacilli.

Collection and Transmission of Specimens, for Examination: Sputum, exudates, urine, spinal fluid and tissues may be examined for tubercle bacilli. They should be collected under as sterile precautions as feasible (not possible with sputum) and transmitted in suitable container to laboratory. Sputum collection should be so guided as to provide bronchial material rather than the fluids from the mouth or nose.

Microscopical Examination (directly applied to specimen): A presumptive diagnosis can be made by applying an acid fast stain, such as Ziehl-Neelson carbo-fuchsin to a slide spread of selected (caseous) fragments of the specimen. The red acid fast bacilli will be readily noted in contrast to the value of the counterstain of all other bacteria cells and debris. Stained spreads may be made from the centrifuged sediment of urine or spinal fluid, using small film of sterile egg albumin on the slide to prevent the sediment being washed off during the staining process.

Concentration Method: If tubercle bacilli are too few to be found by above method they may be concentrated by digesting mucous with sodium hydroxide or antiformin and examining the centrifuged sediment by direct spread, by culture or by guinea pig inoculation.

Sodium Hydroxide Method: Mix equal parts of the specimen and a 3% NaOH solution, shake well, incubate at 37°C for 1/2 hour, add HCl to neutrality to litmus, centrifuge and use sediment for test.

Animal Inoculation: This is the most certain method of establishing the specific diagnosis of tuberculosis. Centrifuge the NaOH digested sputum or the urine or spinal fluid, suspend the sediment in sterile saline and inject this subcutaneously or intramuscularly into the thigh of a young guinea pig (250 gm.). Autopsy of animal at its death several weeks later, or if it lives at 6 weeks, will reveal generalized tuberculosis, apparent particularly by caseation of glands, spotted liver and large spotted spleen, which may be confirmed by finding acid fast bacilli by direct spread or special culture of these tissues.

Cultural Examination: This is not employed as a routine procedure. Several loopsfull of the sediment in the sodium hydroxide concentrate or tissue fragment from guinea pig tissue, are planted on the surface of tubes of Petroff (or other suitable) medium. Incubate the cultures for two days, then seal - use cotton plugs by dipping them in melted paraffin. Incubate at 37°C. for 6 weeks and examine for colonies of M. tuberculosis.

SPUTUM EXAMINATION FOR TUBERCLE BACILLI
(Sodium Hydroxide-alum flocculation Method)

Reagents:

1. Digestor:

Sodium Hydroxide	40.		(4%)
Potassium Alum	2.		(.2%)
Bromthymol blue	.02		(.002%)
Water to	1000.		

Yellow → Blue

Note: Range of indicator 6.0 --- 7.6
pH 7.0 = Light bluish green

2. Acid:

Hydrochloric Acid, Concentration	250.	(25%)
Water to	1000.	(about 2.5 N)

Test:

(1) Mix sputum (5cc.) with 1 to 4 parts of digestor.
Shake well.
Incubate at 37°, 30 minutes for culture on animals.
Incubate at 37° to homogeneous mass for concentration.
(2) Adjust pH to pH 7 with Acid-digestor adjustment.
(3) Centrifuge at top speed for 5 minutes.
(4) Remove supernatant fluids.
(5) a. Spread on slides - heat fix, or
b. Culture or
c. Inject animals with saline suspension of sediment.

Result: the sodium hydroxide digests the organic matter. Flocculation occurs when acid is added. This flocculation carries into sediment the organisms, including Tubercle bacilli, not killed or dissolved by the alkali.

NOTES

SPIROCHETES

Characteristics: Slender, undulating, corkscrew-like, flexible, filamentous organisms. They have short or long spirals with the twists in three dimensions. The number, depth and relative length, and sharpness of angle of spirals have diagnostic importance, though somewhat variable. Motile by sinuous, rotating movement of the body, not by flagella as in the case of bacteria. Stain with difficulty by ordinary stains though some (genus Borrelia) stain readily; the polychrome methylene blue stains of Wright and Giemsa are most used; silver impregnation method is applicable to the more resistant forms. Fontana stain for spreads, Levaditi stain for tissues. Most readily demonstrable, to reveal their characteristic motility, in the fresh state by dark ground illumination. Cultivation difficult and generally not practical. Animal inoculation significant in a few pathogenic species.

Habitat: Ubiquitous, occurring in nature in soil, water, decaying organic materials and on and in the bodies of man, animals and plants. Some are saprophytes, others are commensals, a few are pathogenic, causing such severe diseases as: syphilis, yaws, relapsing fever and infectious jaundice.

BORRELIA RECURRENTIS

(Relapsing fever spirochete)

Characteristics: Spirochetes having large wavy, inconstant spirals, usually about 5; when seen under darkfield illumination, the organisms are very active, in length several times the diameter of an erythrocyte, rapidly progress in either direction, disturbing the red cells by their motion; stain readily and uniformly by polychrome stains (Wright's or Giemsa's) and by simple stains; difficult to cultivate; inoculable into mice and rats, there causing periodicity of spirochetemia but no demonstrable clinical symptoms or tissue lesions.

Habitat: The cause of Relapsing fever; found in blood and tissues of patients suffering from relapsing fever and in the body and intestinal contents of the infector vectors, ticks and lice. The name applies to the spirochete of European relapsing fever; a number of other species--names have been given for the spirochetes of the United States and Mexico (B. turicata), Central and South America (B. venezuelensis) and others, differentiation of which is based only on specific immunological reactions. Some lower animals may serve as reservoirs of infection, in the United States, the armadillo and the opposum.

Identification: Fresh or citrated blood, taken during febrile paraxysm, is examined:

(1) Darkfield illumination of fresh, thin slide-cover glass preparation, for the characteristic motility and morphology.

(2) Slide spread, stained by Giemsa's method or by dilute carbol-fuchsin for morphology. Here the forms are much distorted, the spirals often obliterated, so that the characteristic morphology cannot be found. These spirochetes may sometimes be detected and the diagnosis suggested, in a routine Wright's stain for differential blood count.

(3) White mouse or rat inoculation, intraperitoneal, of .2 to .5 cc. of blood; examine fresh tail blood from the 2nd to 14th day for spirochetes.

FUSOSPIROCHETAL DISEASE

(Vincent's Angina)

Definition: Vincent's angina is an inflammatory lesion in the mouth, pharynx or throat, most often affecting gum margins and tonsils. An acute inflammation may lead to the formation of a pseudomembrane, suggesting that of diphtheria; later there are punched out ulcers, suggestive of those of syphilis. The disease is localized, generally mild with minimal systemic disturbances. Two microorganisms are almost always found together, in great numbers, in films from these lesions; the two forms apparently living in symbiosis. They are rarely present alone, being usually accompanied by other microorganisms, such as staphylococci, streptococci, even diphtheria bacilli; the latter finding being more significant than the Vincent organisms alone.

Fusobacterium plauti-vincenti (fusiform bacillus of Vincent): Large bacilli, thick in middle, tapering toward ends to blunt or sharp points. Readily stained by Loeffler's methylene blue, carbol-fuchsin or Giemsa stain, with characteristic unequality in the intensity of the stain, being more deeply stained near the end; banded alteration of stained or unstained areas in the central body, not unlike the metachromatic granules of diphtheria bacilli.

Borrelia vincentii: Spirochetes somewhat like those of relapsing fever, longer than the fusiform bacilli; made up of variable numbers of undulations, shallow and irregular in their curvatures, unlike the more regularly steep waves of Treponema pallidum. They stain more evenly and less distinctly than the fusiform bacilli.

Identification:

1. Make slide spreads from the ulcerative lesions, fix in flame and stain deeply with dilute carbol-fuchsin, crystal violet or Wright's stain and examine for fusiform bacilli and spirochetes.
2. Positive results will be evidenced by finding great numbers of both fusiform bacilli and spirochetes. A few forms of either type is not significant.

TREPONEMA PALLIDUM

Characteristics: Delicate spirochete coiled in 8 to 14 regular, rigid, sharp spirals; spirals equal or greater in depth than in length, with acute rather than obtuse angles. As seen under darkfield illumination, it appears as a highly refractile, long, slender, spiral, silvery form with serpentine, corkscrewlike movement; motile, but does not progress rapidly or far, motion rotational with undulations.

Made visible most effectively by darkfield illumination. Difficult to stain with analine dyes other than the Giemsa stain; body stained pink by Giemsa stain or black by silver impregnation method, Fontana stain in spreads, Levaditi in tissues.

May be cultured by special methods and inoculated into some lower animals, neither procedure being practical for diagnostic purposes.

Habitat: Strict parasite of humans, causing the infectious disease of syphilis, with protean manifestations; transmitted only by direct contact, generally through sexual intercourse, occasionally through intimate contact of other mucous membrane or skin sites. Syphilis, one of the most prevalent and important of all infectious diseases, usually progresses through a number of states, irregular and varied:

1. Incubation period of 4 to 6 weeks. Spirochetes then cannot be demonstrated.

2. Primary stage: "Hard chancre" at site of inoculation. Starts as a papule, enlarges, becomes hardened and then ulcerates, forming an ulcer, with a firm base and hard edge in typical form, but atypical lesions frequently occur, especially, if mixed with secondary infection or coexistant with chancroid. Spirochetes can be found in fluid expressed from this chancre. The spirochetes will not necessarily be on the surface, rather in the deeper tissues and in the serum exuding from scarified lesion; at this stage they have already become disseminated to a general infection, can be demonstrated in fluid aspirated from satellite lymph gland, but cannot readily be found in the blood or other areas though potentially there.

3. Secondary stage: Characterized by mucous patches, skin rashes and a variety of superficial lesions. Treponema pallidum can usually be found in material from these lesions.

4. Tertiary stage with lesions of viscera, bones, central nervous and cardiovascular systems; tendency to deep rather than superficial lesions. Spirochetes are usually scanty, not readily demonstrated in these lesions.

Identification: (Different procedures applicable to different lesions and stages).

1. Darkfield Examination:

(a) Lesions are cleansed of surface crust, detritus, pus and surface organisms by gauze, or cotton applicator. If lesion has received any germicidal agent, examination is deferred until all germicide has been removed and the lesion has had applied to it, only a saline pack for a day or two.

(b) Primary lesions are then given some trauma, to provoke exudation of serum, by gently rolling the lesions between the gloved finger and thumb or by rubbing its surface with a dry cotton applicator; avoid hemorrhage (though a few erythrocytes or pus cells are desirable to aid in obtaining proper illumination).

(c) Secondary lesions are merely cleansed and abraded.

(d) Slide-cover glass, fresh preparation may be made from accessible lesions by merely touching the slide to tissue juice and immediately placing the cover glass over this moist drop. Vaseline placed around edge will prevent drying. If lesion is less accessible, the fluid may be collected in a capillary pipette and placed on slide from this.

(e) Examine immediately on darkfield microscope for characteristic morphology and motion of Treponema pallidum. Exercise caution not to misinterpret observation. There are many saprophytic spirochetes which are easily distinguished; there are a few spirochetes, especially in the mouth, which are more difficult to distinguish.

(f) "Artifact spirochetes" provoke mistakes to those unfamiliar with the appearance of blood, pus and cultures under darkfield illumination. Wavy filamentous structures may occur there which give a false impression of spirochetes; forms given off by red corpuscles in a drop under a cover glass, may falsely suggest spirochetes.

(g) Report findings with qualifying data, such as notation of location of lesion examined, the occurrence of conditions making examination unrepresentative, etc.

2. Delayed darkfield method: This is a scheme of forwarding lesion fluids to a distant laboratory for darkfield examination; employable when facilities for darkfield examination are not locally available, or when local examiners desire confirmation of their own findings by a consultant. A tissue fluid from a suspected lesion is allowed

to flow into a capillary tube 8 cm. long by 1 mm. diameter; the two ends of this tube are sealed by pressing into a soft paraffin-vaseline mixture (50% of each) and these tubes forwarded for the darkfield examination. At examining laboratory the serum may be transferred to a slide by pressing one end of the capillary tube into a paraffin-vaseline mixture until the opposite end plug is forced out.

3. Nigrosine method: This is not strictly a staining method, for it leaves the unstained spirochete in a black field. A loopful of the fresh tissue fluid is mixed with a loopful of 5% aqueous solution of nigrosine (plus .5% formalin as a preservative); this mixture is spread on a glass slide, dried and examined by ordinary illumination with oil immersion objective. A remote examination may be made by forwarding an air dried drop of the exudate on a slide; the laboratory adds a loopful of water to this to dissolve the exudate and proceeds with the nigrosine preparation. Results are far inferior to the darkfield method, for the characteristic motility is absent and the spirochetes, by distortion, have lost much of their characteristic morphology.

4. India ink method: Like the nigrosine method, a drop of material is mixed with a drop of drawing ink and the mixture spread on a slide. When dry, examine for white spirals against a dark background.

5. Stained spread examination: By Giemsa or Fontana methods.

6. Local Wassermann: Considerable amount of serum is collected from the local lesion and used for complement fixation test.

7. Blood serum and spinal fluid serology: Applicable to later stages. It is customary to subject all venereal patients, even after repeated negative darkfields, to follow-up blood tests for several months.

LEPTOSPIRA ICTEROHAEMORRHAGIAE

(Weil's Disease - Infectious Jaundice)

Characteristics: Spirochetes of many fine coils, so fine as to be difficult to distinguish; one or both ends may be bent into a hook. Rapid spinning motion with intermittent active lashings. Difficult to stain; stained reddish by Giemsa method. Cultivated only by special methods. Inoculable into guinea pig with distinctive lesions.

Habitat: The blood and kidneys of infected wild rats. The blood, urine, kidney, biliary tract of patients with infectious jaundice (Weil's Disease).

Identification:

1. Guinea pig inoculation: Inoculate 3 to 5 cc. of fresh blood, fresh urine sediment or tissue suspension, intraperitoneally into white guinea pig; observe it daily for fever, jaundice in the ears, eyes and about genitalia and for leptospira in the blood (usually found after the 4th day). After the animal dies, large numbers of leptospira can be demonstrated in emulsions of the liver, kidneys and adrenals.

2. Darkfield examination of tissue emulsions, occasionally of urine or biliary sediment, for the motile leptospira.

3. Stained spreads and cultures have limited application.

FUNGI (MOULDS AND YEASTS)

Introduction: The fungi are complex plant organisms, devoid of chlorophyll. The single cell types, as the common budding yeast (*Saccharomyces cerevisiae* grow and multiply much as do bacteria, except as to their method of multiplication (by budding, not by fission). Each individual cell combines the functions of nutrition and reproduction. Other fungi, the moulds, are made up of many cells, usually cylindrical (hyphae), joined into filaments (mycelia) from which spores (small round cells) develop, the structure built up by the filaments and spores being characteristic for each species.

Habitat: Fungi occur widespread in nature and to a less extent in disease. Saprophytic fungi obtain their food from dead plants or decaying materials. Parasitic fungi obtain their food from living animal or plant life. A few species of fungi are pathogenic and capable of producing minor or major skin infections (dermatomycosis), hair infections (trichophytosis), bronchial infections (bronchomycosis) and certain specific generalized infections (blastomycosis, actinomycosis and others). Some fungi have commercial importance, such as those that give flavor to cheese and cause bread to rise (yeast). Many fungi attain laboratory attention because of their ubiquitousness in dust and their contamination of laboratory media.

Descriptive Terms: Fungi occur in many forms, often variable within species under different conditions of growth. Those that are pathogenic tend to grow differently in the tissues than on culture media. Their classification is too complex to be given here. A few terms used in describing and classifying fungi are given below.

Budding fungus: Yeast like; grow by budding; in the tissues and in culture appear as round or oval budding cells; may, but generally do not, develop rudimentary mycelia.

Filamentous fungi: Mould like; develop long filamentous threads with or without apparent spore formation.

Hypha: the single thread-like portion.

Mycelium: a group or matted mass of branching hyphae.

Septa: Divisions of hypha formed by transverse partitions.

Spores: Cells developed for the propagation of the species.

Thallus: The actively growing, vegetative organism as distinguished from spores.

Ascospores: Group of spores, 4 or 8, enclosed in a sac or ascus.

Endospore: A spore formed within an outer envelope.

Conidiophore: Hypha bearing a spore or group of spores.

Blastospore: A spore formed by budding.

Arthrospore: A spore formed of segments of a hypha and released by disarticulation.

Chlamydospore: A large spore with tough and frequently double contoured wall, undergoing encystment.

Sterigma: A short stalk bearing chains of conidia (as in Aspergillus).

Sporangium: A sac containing an indefinite number of spores at the end of a hypha (as in Mucor).

Fuseaux: Fusiform septate spores, produced by certain fungi (Trichophyton).

Spirals: Terminal coils seen in some species.

Pectinate Bodies: Comb-like structures formed by some fungi.

Cryptococcus: A genus of budding fungi devoid of ascospores and mycelia (e.g., Cryptococcus gilchristi, the causative organism of one form of blastomycosis).

Saccharomyces: A genus of budding fungi having ascospores but no mycelia (e.g., Saccharomyces cerevisiae, brewers yeast, oval or spherical cells, cause alcoholic fermentation).

Monilia: A genus of budding fungi having no ascospores, mycelia of rudimentary type and capable of fermenting certain sugars with the production of acid and gas. (e.g., Monilia psilosis, formerly thought to cause sprue).

Endomyces: A genus of budding fungi, having ascospores and segmented mycelia (e.g., Endomyces albicans, found in thrush).

Madurella: A genus of filamentous fungi characterized by septate, branching hyphae and chlamydospores; they are contained in black granules of infected tissue (e.g., "Madura Foot") (e.g., Madurella mycetomi in "Madura Foot".)

Nocardia (Actinomyces): A genus of filamentous fungi characterized by very fine, non-segmented mycelial filaments and no spores (e.g., Nocardia bovis, the "ray fungus" of "lumpy jaw" of cattle, actinomycosis of man).

Sporotrichum: A genus of filamentous fungi which appear in the tissues as oval spores and develop in cultures as mycelium with characteristic grouped spores or conidia. (e.g., Sporotrichum schenki, the causative agent of sporotrichosis, appearing in fresh spreads of the pus or tissue as oval or cigar shaped cells, and when examined by hanging drop culture appear as fine interlacing septate hyphae with oval or pear shaped spores, attached to the hyphae.)

Aspergillus: A genus of filamentous fungi characterized by its spore organ. They are common and troublesome laboratory contaminants, appearing in

culture plates as cottony masses dotted with minute colored spots, becoming in older cultures profusely black, yellow or green, according to the species. Microscopically, the colored spots are seen to be the spore organs, the spores (conidia) borne on aerial hyphae which terminate in a large rounded head with rows of spores projecting in all directions. The main cottony mass is a network of septated mycelial filaments. (e.g., Aspergillus niger, the black mould.)

Penicillium: This genus differs in its spore organ in that the fertile hyphae show numerous branches, rather than a rounded head, bearing rows of spores, a structure somewhat resembling a broom. The color of the colony varies with the species - green, yellow, etc. A common variety is Penicillium brevicaule and its strains, it causes spoilage of cheese and other dairy products.

Phycomycetes: A group of genera having, in addition to the mycelium, spores contained in a spherical, case-like structure (a sporangium) at the end of a hypha. Species of this group frequently contaminate laboratory media and food products, and occur in soil, dust and water. (e.g., Mucor mucedo, the blue-black mould.)

Pleomorphism: This term refers to the great variation in characteristics of morphology and culture which many fungi undergo under different conditions of life. The ringworm group are particularly likely to undergo these degenerative changes, and once a culture has so changed, it cannot easily be restored to its original condition. Prolonged growth on sugar-containing media leads to this permanent change, hence the use of "conservation agar" for stock culture maintenance.

Ringworm Group of Fungi: These filamentous fungi produce superficial skin infections, generally growing as leathery masses of closely interwoven hyphae, growing slowly with development of bumps and ridges, and covered by a powdery or velvety "duvet" of aerial hyphae. The next four genera belong in this group. The so-called "Athlete's foot" may be caused by the same group of fungi.

Microsporum: The small spored ringworm fungus: in the diseased epidermis they appear as a fine mycelium, one to five microns in diameter, composed of rectangular elements; they penetrate into the hairs and grow up and down in the hair. When the infected hairs are examined, they are found to be encased with an irregular mosaic of small round spores about 2 microns in diameter. (e.g., Microsporum audouini, causing the common ringworm of the scalp.)

Trichophyton: The large spored ringworm fungus: the mycelium consists of chains of oval or rectangular spore-like bodies 5 to 8 microns in diameter, in regular alignment. Various species commonly produce ringworm of the scalp, beard, skin and nails. (e.g., Trichophyton tonsurans, which is found only within the medulla of the hair.)

Epidermophyton: A genus of skin-invading fungi, appearing as long interlacing filaments, never invading the hair as do trichophytons. May be readily recognized by direct microscopic examination of skin scraping. (e.g., Epidermophyton cruris, causing the common "Dhobie Itch" or ringworm of the groin and other areas.)

Achorin: A genus of filamentous fungi of which a species causes favus of scalp (Achorion schoenleini.)

Materials for Examination:

1. Hairs, skin scales or scrapings from lesions.
2. Tissue masses or scrapings from internal lesions.
3. Secretions or excretions from infected areas.
4. Incidental cultures (contaminants, etc.).

Methods of Examination

1. Choice of Method: Varies with expectancy of findings. Skin scrapings and hair may yield informative data by immediate direct methods only and be less informative on culture or animal inoculation, being difficult to grow, to infect or to exactly identify. Some species give the desired information only on cultivation or animal inoculation, especially in the case of tissue invaders. A few pathogens may be detected by histopathological examination.

2. Microscopic examination:

a. Collection of specimen: (preferably in laboratory by a medical officer). After cleansing the affected part with alcohol, such materials as hairs, nails, scales or bits of tissue may be scraped into a sterile Petri dish. Moist specimens should be prepared and examined without delay. Biopsy specimens should be divided, one-half for direct examination, the other half for fixation and histological examination. All specimens should be obtained from an active infected area and not from dried or inactive lesions. Collection of sputum for this examination requires especial care to avoid mouth contaminants by previously rinsing mouth with sterile saline solution, the expectoration then to be placed in a sterile Petri dish and examined within a few hours.

b. Fresh preparation for direct study: outline a vaseline circle on a slide, place the material under examination within this circle, add a few drops of 10% sodium hydroxide, cover with a cover glass and examine after a period of digestion - a few minutes to twelve hours. Fungi resist the digestive action of the hydroxide and retain their form, whereas tissue elements disappear. Avoid mistaking artifacts, resembling yeast-like organisms and hyphae.

c. Stained spreads: Moist materials may be spread on a slide as for bacteriological study, for bacterial stains and for a polychrome (Wright or Giemsa) stain. The former stains will reveal bacterial content, the latter will assist in the study of any fungi present but will also bring out cellular detail and may lead to the discovery of a protozoan infection as leishmaniasis.

3. Cultivation: Seventy per cent alcohol may be used to cleanse scales or hair of contaminating bacteria, by allowing the specimens to soak therein for about one hour prior to placing them on culture media (control culture to be made of the alcohol to detect contaminating spores in it). Materials, with or without above preparation, should be placed upon media having a pH of 5 to 6, unfavorable for bacterial growth. Inoculate several Sabouraud's maltose agar tubes, planting the inoculum into a slightly broken surface of the slant. Incubate at room temperature (22°C.) and at 37°C. Many fungi show cultural differences when grown at 22°C and at 37°C. Retain the cultures for at least four weeks before considering them negative.

Observe the cultures daily, but do not open the tubes unless definite growth is observed, then make a subculture as soon as the tube is opened. Subcultures are made on Sabouraud's maltose agar or on his conservation agar; the former is best for primary isolation, the latter for storage and the study of characteristics. In studying the yeast-like organisms, corn meal agar plates are useful for isolation, purification and low-power study under the microscope. These are best inoculated by so streaking the plates that the wire produces a slit in the medium.

Cultures may be studied by observing colony characteristics by naked eye, under low power magnification and by slide preparations, fresh or stained, for microscopic examination. Instead of removing, as for bacterial spread examination, a surface loopful of the colony, it is best to remove, with a stiff wire, a fragment of the culture supported intact in a fragment of the culture media; this is placed in a drop of lacto-phenol (equal parts of lactic acid and phenol) or of water, on a slide, and covered with a cover glass. In studying these slides the microscope light is modified to provide a subdued light (by lowering of substage and control of diaphragm). Study hyphae, branching, budding, sporulation, septation, etc., for descriptive report of the cultured fungus.

Hanging drop cultures in maltose broth, afford another method of study.

4. Animal Inoculation: Most of the fungi are not pathogenic for laboratory animals. A few species have animal pathogenicity and animals may therefore be used in determining the characteristics of only those few species. The form of the fungi seen in tissues tends to be quite different from the form seen in cultures. Some of the yeast-like fungi, producing as a group, blastomycosis, are pathogenic for animals: diagnostic information is, in these obtainable by the subcutaneous, intramuscular, intraperitoneal or intravenous inoculation of the suspected material, or preferably, a pure culture of the isolated fungus. The mouse, rat, guinea pig, or rabbit may be so used, and observed for a prolonged period for local or general evidences of infection.

THE RICKETTSIAE

Description: Extremely small bacterium-like organisms, varying from just-visible coccobacilli to filaments 1.5 to 2.0 microns long; found in the alimentary tract of certain Arthropods, and in the tissues of man and animals during a variety of diseases of which they are the causative agents; usually non-motile; Gram-negative, but stain poorly with usual dyes; Giemsa stain applied for 10-24 hours is preferred stain for Rickettsiae in infected tissues, with which stain they appear as purple dots, or bipolar staining bacilli, packed within tissue cells, or extracellularly; fail to grow on ordinary culture media, but may be grown in tissue cultures and in living chick-embryo cultures.

Habitat: Many species, all of which are strictly parasitic on Arthropods, animals and/or man; the cause of five more or less clearly defined groups of diseases in man, many of which are primarily diseases of rodents, or other animals, being transmitted to man by the bite of infected lice, ticks, mites, or fleas; no or only slight cross-immunity produced between groups. They are found in the blood and all the organs of man or other infected animal, but show a preference for mesothelial cells, especially those cells of the tunica vaginalis, peritoneal cavity and intima of small blood vessels.

1. Typhus fever group: Consists of two types of disease which immunize one against the other; the rickettsiae always develop within the cytoplasm of infected cells; transmitted by lice and fleas;
   - a. Epidemic Typhus is transmitted from man to man by the body louse (*Pediculus humanus var. corporis*). Infective agent - *Rickettsia prowazeki*.
   - b. Endemic typhus is normally a pathogen of rats and other rodents and is transmitted from rat to rat and rat to man by rat fleas (*X. cheopis* and *C. fasciatus*)· however, during epidemics in man, it may be transmitted from man to man by the body louse. Infective agent - *Rickettsia mooseri*.

2. Rocky Mountain Spotted Fever Group: Consists of two types of disease found in the United States, "Western type" and "Eastern type" R.M.S.F. and several identical or closely related diseases under different names in other parts of the world, which immunize one against the others; the rickettsia often are found packed within the nucleus of infected cells; transmitted by ticks, which, when once infected, are capable of transmitting the virus through the egg to their offspring; vector for "Western type" is *Dermacentor andersoni* and for "Eastern type" is *D. variabilis*. Each type is caused by strains of the same virus (*Rickettsia rickettsi*).

3. Other groups:
   - a. Tsutsugamushi fever group: Found in Japan and other oriental countries (Tsutsugamushi in Japan, Rural or Scrub typhus in Malaya, and "Mite fever" in Sumatra); causative agent - *R. nipponica*; transmitted by bite of infected larval mite (genus *Trombicula*).

b. Trench fever: Epidemic amongst troops on all fronts in Europe during World War I; causative agent - R. quintana; transmitted by body louse.

c. "Q" fever: Found in Australia and western U. S.; causative agent - R. burneti; vectors not yet determined, but the tick (D. andersoni) has been incriminated in the U. S.

Collection and Transmission of Specimens for Examination:

1. Clear, sterile serum of patient for Weil-Felix agglutination test.

2. Sterile defibrinated or citrated blood for animal inoculations.

3. Autopsy material, fresh and fixed in formalin, such as portions for brain, spleen and tunica vaginalis, for animal inoculation and for sectioning.

Weil-Felix Reaction: This is the most reliable diagnostic test for identifying fevers due to rickettsiae. It is based upon the fact that, in patients with this group of fevers, agglutinins which react with certain varieties of Proteus organisms develop in the patient's serum after the fourth day; in the case of typhus fever, agglutinations as high as 1/40,000 have been reported; a titer of 1/100 or higher is usually considered as diagnostic; see table for interpretation of results. The reaction is based upon a macroscopic test-tube agglutination test, using a series of dilutions of the patient's serum against Proteus antigens.

The Proteus strains, used, were originally isolated from typhus fever patients, but apparently had no connection with the disease. The X19, X2, and "Kingsbury" strains have been found to be of the greatest value. Since the antigens common to the Proteus strains and the rickettsiae are found in the body of the bacilli, the antigens, used, should be prepared from non-motile "O" variants, preferably heat killed or alcohol treated. The antigens are then designated as OX19, OX2 and OXK.

Animal Inoculations: Guinea pigs, rabbits and monkeys are susceptible to many of these viruses when inoculated intraperitoneally with 3 to 5 cc. of blood, or of suspension of brain or other tissues. The animal of choice for inoculation is a nearly grown, male guinea pig to attain the characteristic scrotal lesions. Cross immunity tests by inoculating several guinea pigs, each protected against a specific virus, are of great differential diagnostic value. In addition to reactions given below, proof of type disease depends upon (1) transfer of infection to second guinea pig and (2) sterility of ordinary bacterial cultures from the organs.

Reactions in the guinea pig:

1. Epidemic typhus: Incubation period, 5 to 14 days; temperature of 103.5 to 106°F. for 3 to 14 days; no gross scrotal or testicular lesions; brain shows small proliferative nodules, with perivascular infiltration; guinea pig usually recovers; a solid immunity is produced, also protecting against endemic typhus.

2. Endemic typhus: Incubation period, 4 to 6 days; temperature of 103 to 105°F. lasting for 3 to 5 days, showing a saddle back curve, with a drop during the second day of fever; scrotal lesions, erythema and swelling of the scrotal skin, the tunica vaginalis is adherent and Giesma-stained scrapings will show the presence of numerous intracytoplasmic rickettsiae; brain lesions, similar to but less marked than epidemic typhus; the guinea pig never dies: immunity, same as for epidemic typhus.

3. Rocky Mountain Spotted Fever, Western type: Incubation time, 2 to 4 days; temperature rises rapidly to 106°F.; scrotal lesions, erythema and edema, commonly accompanied by necrosis; brain lesions, same as for epidemic typhus; 90 to 100% of guinea pigs die within week to 10 days; recovered animals are immune to both types of R.M.S.F. but not to typhus or other groups.

4. Rocky Mountain Spotted Fever, Eastern type: Incubation time, 3 to 5 days; temperature, 104 to 106°F.; scrotal lesions, erythema and edema, no necrosis; brain lesions present, but sparse; mortality 25 to 30% immunity, same as for Western type.

5. "Q" fever: Incubation time, 4 to 6 days; temperature, 103 to 106°F.; no scrotal or brain lesions, the chief post mortem finding being an enlarged spleen; frequently kills animal; specific immunity produced.

6. Tsutsugamushi and Trench Fever Groups: Not infective, or produce only slight rise in temperature.

Cultural Examination: This procedure is not routinely employed. The rickettsiae will not grow on usual media, or in the absence of susceptible tissues, where they develop within the cells; after passage through guinea pigs, they may be cultured (1) in a modified Maitland's medium containing 20% guinea pig or horse serum and sterile minced tissue, such as the tunica vaginalis of guinea pig, or mouse embryo, or (2) in living chick-embryo tissues by direct inoculation into the yolk of a sterile egg after 5 to 10 days incubation.

Microscopical Examination: Not usually made; rickettsiae may be found in small numbers in the blood and various tissues but their demonstration is of no practical differential value.

Table - INTERPRETATION OF LABORATORY TESTS FOR RICKETTSIAL DISEASES

Disease	Weil-Felix Reaction			Results of Guinea pig inoculations	
	OX19	OX2	OXK	Scrotal lesions	Brain lesions
Epidemic typhus	+++	+	---	---	++
Endemic typhus	+++	+	---	Erythema, edema	+
Western Spotted Fever	+	+	+	Erythema, edema Necrosis	++
Eastern Spotted Fever	+	+	+	Erythema, edema	Sparse
"Q" Fever	---	---	---	---	---
Tropical Scrub Typhus	---	---	+++	Not infective	Not infective
Tsutsugamuchi	---	---	+++	Not infective	Not infective
Trench Fever	?	?	?	Not infective	Not infective

CULTURE MEDIA

I. Preparation and Sterilization of Glassware.

A. Cleaning: one of the most important steps in the preparation of culture media is clean glassware. There are three kinds of clean glassware.

1. Physically cleaned glassware: this is done by washing the glassware with soap and water. New glassware may be soaked in warm soap water for an hour or two, rinsed in tap water several times, and finally rinsed in distilled water. Most used glassware may be washed in the same manner. If there is anything on the tubes or flasks that won't come off by simple washing, then it is necessary to put it in dichromate cleaning solution.

   The glassware should be placed in a soap solution. The soap solution is prepared in a sink by taking a rather large can and punching holes with a knife in the bottom and lower 1/3. Yellow GI soap is sliced into small pieces and hot water is run through the can of soap. After the desired amount of water is drawn, the solution can be made as soapy as desired. Before putting dirty glassware into this soap water, all agar, blood, etc. should be rinsed out with tap water. This glassware is then placed in the soap water for an hour, then cleaned with a brush. There is a variety of brushes available, and one can be found to clean any kind of glassware.

   Heavy wax pencil marking may be removed with scouring soap or powders. Scorched agar and dye stains are removed by the bichromate cleaning fluid. Paraffin may be removed by xylol or placed in the cresol solution. Adhesive tape can be removed by ether or xylol.

   A very small amount of glacial acetic acid may be added to the distilled water used for rinsing the glassware. After the glassware has been run through the acidulated distilled H_2O, it is placed in wire baskets or small packing boxes and dried in the hot air sterilizer or allowed to drain dry. Physically cleaned glassware will answer most purposes of culture media.

2. Chemically Cleaned Glassware: this is glassware especially prepared for use where the presence of a foreign chemical may be detrimental to a test.

   All spinal fluid tubes (tubes which are to be used to draw spinal fluid specimens); pipettes (especially those used for titration media) must be cleaned chemically. This is done by leaving them in the dichromate cleaning solution overnight, or longer. The glassware is removed from this solution, rinsed in tap water; distilled water, then boiled for one hour in distilled water. The tubes or pipettes are then dried.

   Bichromate cleaning solution; potassium (or sodium) bichromate, technical - 100 gms.; concentrated sulfuric acid (either technical or commercial) - 250 cc.; distilled water - 750 cc. Dissolve the potassium bichromate in the water and add the acid very slowly.

Extreme care must be exercised due to the heat generated by the addition of the acid. It is best to make the solution in a 2000 m. pyrex flask.

3. Bacteriologically Cleaned Glassware: this is glassware that has had all cultures or contaminated materials destroyed. This is done by placing them in a large container which has a 2-5% solution of saponated cresol for 24 hours, or placing in an autoclave and sterilizing for thirty minutes at a 15 lb. pressure.

   Rubber gloves should be worn while removing the glassware from the cresol, as it will burn the hands after a few minutes unprotected. Care must be taken in handling the glassware, because it is very slippery when it comes out of the cresol.

   After the glassware has been left in the cresol long enough, place it all into the sink, rinse the cresol off thoroughly and remove all the agar in tubes by means of a small test tube brush, which has been used for some time and is partially worn out. <u>All traces of agar must be removed from tubes, Petri dishes and flasks</u>.

B. Sterilization of Glassware.

Sterile glassware is essential for accurate work in Bacteriology, since it would be difficult to identify an organism in the mixed contamination caused by unsterile glassware.

Whereas, in the vegetative form, many spore-bearing organisms are easily destroyed; some of the spores are very resistant, so this must be taken into consideration.

Heat, both dry and steam, is used for sterilizing. Hot air sterilizing is preferable. Glassware may be sterilized with steam under pressure (that is, in the autoclave) at a 15 or 20 pound pressure for from 15 to 30 minutes, then placing in the hot air sterilizer to dry.

The Hot Air Sterilizer

This type of sterilizer is similar to a detached oven of a gasoline or oil stove. It is double-walled and usually gas is the source of heat. A sterilizer with some sort of adjustment for heat should be used. One or two holes should be provided for thermometer, and a constant vigilance maintained until the proper temperature is obtained. Two or three metal shelves should be used in the sterilizer.

The temperature may run from 180° C. - 200° C., 185°C. being an ideal temperature. Following is a general time for sterilizing and drying various things:

170° C.- 180° C.

Item	Minimum Time	Maximum Time
Test tubes, flasks, vaccine bottles, needles, glass stoppered	2 1/2 hrs.	3 1/2 hrs.
bottles, Petri dishes	4 hrs.	- -
syringes and wrapped pipettes	3 hrs.	4 hrs.

For drying: about the same length of time, except Petri dishes, which will dry in about 30 to 45 minutes, and pipettes, which should be left dry at least 4 hours or more.

180° C. - 190° C.

Test tubes, flasks, vaccine bottles, etc.	1 hr. 45 min.,	2 hrs. 15 min.
Petri dishes	3 1/2 hrs.	- -
Syringes and pipettes (wrapped)	2 1/2 hrs.	3 hrs.
For drying:		
Tubes and flasks	1 hr.	1 1/2 hrs.
Bottles	2 hrs.	3 hrs.
Petri dishes	30 min.	- -
Pipettes	3 hrs.	- -

190° C. - 200° C.

Caution: Cotton and paper and cloth disintegrate at temperatures above 190° C., so when sterilizing, watch your glassware carefully.

Test tubes, flasks, needles, etc.	1 hr.	1 1/2 hrs.
Petri dishes	2 1/2 hrs.	- -
Syringes and Pipettes (wrapped)	2 hrs.	2 1/2 hrs.
For drying:		
Tubes and flasks	45 min.	- -
Bottles	1 hr.	1 1/2 hrs.
Petri dishes	15-20 min.	- -
Pipettes	2 hrs.	3 hrs.

Cotton plugs in test tubes should be a cream or light tan color when sterile. Cloth should be light tan to a light brown.

Petri dishes may be placed on the top shelf of the sterilizer. Avoid putting dripping glassware into the sterilizer in such a manner as to allow water to drop on hot glassware. If a wooden or cardboard box is used as a container for drying glassware, it should be placed on the bottom away from the sides of the sterilizer, if the temperature exceeds 190° C. Tubes containing cork stoppers should also be placed on the lower shelf of the sterilizer when temperature is 190°C.

C. Preparation of Glassware for Sterilization.

1. Syringes.

   a. 30 cc: 30 cc syringes are sterilized for the purpose of drawing blood for blood agar, "chocolate agar", and blood for typing serum.

      The syringe and needle are prepared together. After the needles have been well sharpened, put them on the syringe tightly with pliers, being sure the bevel of the needle is facing up with the graduations on the barrel of the syringe. Then the stylet is inserted and a Wasserman tube is put over the needle and stylet for protection.

      Next a piece of unbleached muslin about 1 foot square is cut and laid down on a table. Place the syringe diagonally and roll up in the muslin securely, tucking in the corners. Then tie a string around it, and it is ready for sterilizing.

   b. 10 cc: the 10 cc syringe is prepared the same as a 30 cc, only, instead of wrapping in muslin, the syringe may be placed in a large test tube and muslin (about 4 inches square) tied over the top of the syringe and tube.

      The syringe and needle may be sterilized separately. Then, wrap the syringe in muslin, and put the needle with a stylet point down in a Wasserman tube, plug with cotton and sterilize. Needles used for taking Wassermans, blood chemistry, etc., are prepared in the same manner.

2. Pipettes: pipettes may be sterilized in metal cans made for that purpose, or they may be wrapped in paper individually and sterilized. Toilet tissue is an excellent paper to wrap pipettes in.

3. Flasks: Erlenmeyer flasks of all sizes may be sterilized by taking a piece of cotton and making it into a roll, according to the size of the flask. Place this cotton roll in a piece of gauze from 3 inches to 6 inches square, then insert into the mouth of the flask.

4. Test Tubes: test tubes are plugged with Grade A cotton. There are several methods of doing this but I personally have found that the best method is to roll the plugs. This is done by taking a piece of cotton about 2 1/2 inches wide and about 3 inches long; fold the cotton so that it is about 1 1/4 inches wide and 1/4 inch thick. Then roll the cotton rather tightly and insert into tube. This method is somewhat difficult at first but with practice, a good durable plug can be made. All test tubes, and vacinne bottles are plugged in this manner.

5. Bottles.

   a. Wide-mouthed specimen bottles are plugged either with gauze and cotton or with a #20 cork stopper, and a piece of muslin over the top. These bottles are used for stomach contents, sputum, and for shipping specimens from one station to another.

b. 6 oz. glass-stoppered bottles are provided for the shipment of milk and water specimens for bacteriological examination. A piece of muslin about 4 inches square is tied securely over the top of the bottle.

c. Small vials are prepared for feces by inserting a small metal paddle in a cork and putting it in the vial and sterilizing.

d. 240 cc. glass-stoppered bottles are sometimes used for the shipment of food for examination. These are sterilized in the same manner as the 6 oz. bottle. All test tubes, flasks, pipettes, syringes, etc., must be absolutely dry before being sterilized.

II. Preparation of Culture Media.

A. General Considerations: almost all the work done in bacteriology depends entirely upon culture media.

1. What is Culture Media?

Culture media is artificial food for bacteria. It is composed of organic substances and inorganic or minerals.. Of the organic we have chiefly a nitrogenous substance called peptone, meat extract, blood, serum, eggs, agar and many others. Minerals used are various salts, such as sodium chloride, potassium phosphate, sodium citrate and others.

Now bacteria are very exacting in their food requirements, more so than human beings, because if a person is hungry, he will eat almost any food set before him. But bacteria are different, some will grow on one thing in which another family of bacteria can't possibly live.

So, if the bacteriologist is to do accurate work, he must have a wide choice of well prepared media to work with.

B. Determination of the pH of Media Titration.

1. pH is the log reciprocal of the hydrogen ion concentration, or to put it simply, it means the acidity or alkalinity of a substance. The pH scale is zero to fourteen. Seven being neutral. Above seven is alkaline or basic. Below seven is acid.

Bacteria have what is called their "optimum pH." If the pH of the medium deviates from the optimum pH of the bacteria to too great an extent, the growth of the bacteria will be inhibited or stopped altogether.

Most bacteria grow best in a slightly alkaline medium. It is my belief that pH is the most important consideration of Culture Media.

The technic most commonly used in the adjustment of the pH of media is the colorimetric method.

2. Reagents, equipment and apparatus required.
   a. Pipettes.
      (1) 10 cc. graduated in 0.1's; two or more; 1 cc. graduated in 0.01's.
   b. One 25 cc. graduate, graduated in cc's.
   c. Culture tubes, holding approximately 20 cc.
   d. pH standards: Phenol - red standards are used. pH 6.8; pH 7.0; pH 7.2; pH 7.4; pH 7.6; pH 7.8; pH 8.0; pH 8.2; and pH 8.4
   e. Phenol-red indicator. 0.02% aqueous solution.

      An indicator is a substance, usually a dye, which has the ability to change its color progressively as the pH changes. For example, phenol red, when in an acid solution is lemon yellow. At pH 6.8, it has a slight pink color. At pH 7.0, it has a definite pink color, and as the pH rises to pH 8.4, it becomes pinker until it is red. Indicators have what is called their "range," that is, where the color change begins and ends. The range of phenol-red, is pH 6.8 - pH 8.4. The phenol-red standards may be obtained from the Army Medical School at Washington.
   f. Normal NaOH.
   g. Distilled water.
   h. A wooden block painted black, 3 inches long, 2 inches wide and 2 inches high, with four holes large enough to accomodate your standard and three culture tubes, and four holes in the side about 3/4 to 1 inch apart, two on either side of the block permitting one to see through the block.

3. Procedure:

a. Take the 25 cc graduate and get exactly 19 cc of distilled water. Add one cc of normal NaOH. Mix thoroughly by drawing the distilled water into the 1 cc pipette and blowing it out. Repeat this several times. This gives you N/20 (1/20 normal) NaOH.

b. In the back row of two holes in the block, place the following: on the left, a tube with 10 cc of distilled water; on the right, your pH standard, for example: pH 7.6 standard.

c. Place two empty tubes in front of the distilled water and standard. In the tube in front of the pH standard, put 10 cc of the media that is being tested.

In the other tube put 9.5 cc of media and 0.5 cc phenol red indicator. Mix by inverting one time. If the tip of the thumb is dipped into the medium, it will have the same reaction and the contents of the tube will be unaffected.

d. Compare the color of the medium and the indicator in the one tube with the color of the standard and other tube together.

e. If the colors do not match, the medium is too acid. Slowly add N/20 NaOH, inverting from time to time to mix. When the color on the left compares with right, then, you are ready for the next step:

Let us assume that we have 8000 cc of media. If it takes 1.3 cc of N/20 NaOH for 10 cc of media, then for 8000 cc it would take:

10 cc - - 1.3 cc
100 cc - - 13 cc
1000 cc - - 130 cc
8000 cc - - 1040 cc of N/20 NaOH

Obviously we cannot add 1040 cc because it would dilute the media, so we calculate the amount of <u>normal</u> NaOH. That is done by dividing 1040 cc by 20:

$$20 \overline{)1040 \text{ cc}} = 52 \text{ cc}$$

Hence, 52 cc N NaOH is added to the medium and thoroughly mixed. Then the pH is checked. If the reaction isn't satisfactory, repeat the titration until it is. The pH is usually adjusted at or near the boiling of the medium. Usually, during sterilization of media, the reaction changes somewhat, so about 0.2 pH should be allowed.

C. Clarification: Media must be clarified. This is done by centrifuging, by filtration, by coagulants, and by decanting. All broths may be filtered through paper. All agar is filtered through cotton and gauze.

D. Distribution.

1. The stock media is kept in Erlenmeyer flasks. The 1 liter flasks containing 600 cc; the 2 liter flasks 1500 cc; and 500 cc. flasks 300 cc.
2. Tubing of media is done by placing in a large container or funnel to which is attached a rubber hose and controlled by a pinchcock.
3. For agar slants, about 5 cc; for unslanted agar, 10 to 15 cc; and for fluid media 10 cc. Agar media must not be allowed to cool too much while tubing.

E. Storage: after sterilization, the culture media should be kept in a refrigerator. Paper should be tied over the neck of flasks or lead foil may be used. This prevents the entrance of fungi and contaminating of the contents.

F. Sterilization of Culture Media: all media must be sterilized; this is usually done in an autoclave, or with steam as in the Arnold Sterilizer, by filtration through a Berkfeld Filter, in an inspissator, or by boiling water bath. All the stock agars and broths are sterilized at 15 lb. pressure for 15 minutes. Most sugar broths are sterilized at 10 lb. pressure for 10-15 minutes.

1. Fractional or intermittent sterilization:
   a. Media is sterilized for 3 successive days either by boiling or in the Arnold sterilizer, for from 30 to 45 minutes.
   b. On the first day of sterilizing, the vegetative forms of bacteria are killed, and some spore forms are activated. On the second day, those spores activated from the first day have been changed to the vegetative forms. These are killed by the second sterilizing. The most resistant spores are activated and on the third day, the vegetative forms of the most resistant organisms are destroyed. This may be illustrated in the following chart:

		Vegetative Form		Spore Form
1st Day		V		S
2nd Day		V		S(Most re-
3rd Day		V		sistant spores)

G. Pour-Plates: are media distributed in Petri dishes, such as blood agar plates, EMB plates, etc. All the plates are poured aseptically. 10-20 cc. of agar is poured. Enough, so that the media will be about 1/8 inch thick. This is done by first flaming the neck of the flask containing the agar. Raise the top of the Petri dish in such a way that one edge is touching the table top, then pour the desired amount.

While the agar is still warm, flame the entire surface of the plate to destroy what bubbles may be present.

H. During the process of cooking media we figure 25% of the volume to be lost through evaporation. There are two ways of taking care of this loss.
   1. Either make a mark on the side of the pot or measure with a stick and add distilled water to the original volume.
   2. The other method is to take 25% of the volume and add to the original volume before you start cooking. For instance, if the volume of media being prepared is 8000 cc, then 25% of this would be 2000 cc. This 2000 cc is added to the 8000 cc. About 100-125 cc will boil off in one minute, so you would boil vigorously for about 20 minutes.

I. Differential Media: Many groups of organisms are very similar and can only be identified by certain reactions on various media. This media is called "differential media" and includes the following:
   1. Sugar Broths.
   2. Russell's Double Sugar.
   3. Simmon's Citrate Agar.
   4. EMB agar.
   5. Clark and Lub's Medium.
   6. Jordan's tartrate agar and others.

   These various media are especially useful in differentiating organisms of the colon - Typhoid - Dysentery Groups. Usually all of the above media is used with the same organis, and identification is clinched.

J. Solid Media: solid media is prepared with agar, gelatin, or coagulated eggs or serum. Almost every broth made up, there is a corresponding agar, for instance, beef extract broth, and beef extract agar. Agar is the word used both for the medium and for the substance which makes the medium solid. Agar is made from a certain species of Asiatic seaweed; it is a glue-like substance. It may be dissolved in hot water, and when cooled forms a solid. From 1½ to 3% is usually used.

K. Liquid Media: Liquid media is the various broths prepared.

L. Semisolid media: This is prepared by adding a small amount of agar or gelatin. (0.5% agar)

Now I will give you some formulae commonly used in routine bacteriological work:

III. Formulae.

A. Broths: Beef extract broth (Meat) (For routine use).

1.	Beef extract	3 gms.
2.	Peptone	10 gms.
3.	Sodium Chloride	5 gms.
4.	Distilled water	1000 cc.

Add the weighed ingredients to the distilled water and dissolve manually or by heat. Adjust the reaction so that the final pH will be between 7.4 - 7.6 filter through paper and distribute in tubes or flasks. Sterilize 16 lbs. pressure for 15 minutes.

Nutrient Broth
(Standard for water analysis)

Beef extract	3 gms.
Peptone	5 gms.
Distilled H_2O	1000 cc..

Prepare as directed above. Adjust reaction between pH 6.4 and 7.

Meat Infusion Broth

Beef or veal round, free from fat, ground	500 gms.
Distilled water	1000 cc.

Mix the meat and water and infuse in the icebox for 18-24 hours. Heat in a boiling water bath over a low flame for about 1 hour. Filter through a cotton and gauze filter. Add 5 gms. of sodium chloride and 10 gms. peptone to 1 liter of the broth. Adjust the reaction to pH 7.8, and prepare in the same manner as beef extract broth.

Lactose Broth
(Standard for water analysis)
To 1000 cc of nutrient broth, add 5 gms. lactose (0.5%); dissolve tube in a fermentation tube, and sterilize in autoclave 15 lbs. for 15 minutes.

Clark and Lub's Medium
(For Voges-Proskauer and Methyl-Red Tests).

1. Peptone (Difco Proteose	5 gms.
2. Dextrose (CP)	5 gms.
3. Potassium Phosphate K_2HPo_4	5 gms.
4. Distilled water	1000 cc.

Dissolve 1, 2 and 3 in 4 with heat. Filter and tube in Loeffler tubes. Sterilize by boiling in a water bath 30 minutes on three successive days.

Brain Broth or Blood Culture Medium
(for blood cultures).

1 calf brain	
Dextrose	10 gm.
Sodium Citrate	10 gm.
Infusion broth (pH 7.4 - 7.6)	1000 cc.

Dissolve the dextrose and sodium citrate in the infusion broth. Filter, wash and remove all membrane and blood from the brain and chop it into pieces as large as the end of your thumb. Wash a number of marble chips the size of the end of a finger. Place the marble chip and piece of brain in a large tube and the filtered broth with the dextrose and sodium citrate. Sterilize 15 lbs. for 15 minutes.

Sugar Broths

1. Dextrose
2. Maltose
3. Mannit
4. Xylose
5. Arabinose
6. Lactose
7. Saccharose
8. Dulcitol
9. Inositol
10. Inulin

To 100 cc. of beef extract broth pH 7.4 - 7.6, add 0.5 gm. of the desired sugar, and 0.1 cc. of Brom-Cresol-Purple indicator 1.6% alcoholic solution. Tube in small fermentation tubes 5-10 cc. Sterilize by boiling in water bath 30 minutes for 3 days. Or sterilize in the autoclave at a pressure not exceeding 7 lbs. for 10 minutes.

B. Agars: Sterilize all agars 15 lbs. for 15 minutes, unless otherwise stated.

Beef Extract Agar
(For routine use)

To 1000 cc. of Beef Extract Broth, add 20 gm. of agar. Heat the broth to about 80°C., before adding the agar. Stir constantly while the agar is being added to prevent lumping and scorching. When the media begins to boil, titrate. Adjust the reaction to pH 7.6. Filter through a cotton and gauze filter. This is stock medium and is used for plain agar plates and slants, and as bases of blood agar and Russell's double sugar. After filtering, the agar should be distributed in 500 cc. and 1 liter flasks. Put 300 cc. of agar in the 500 cc. flask, and 600 cc. in the one liter flask.

Levine's Eosin - Methylene - Blue Agar (EMB)
(Standard for water analysis)

1.	Pepton (Difco)	10 gm.
2.	K_2HPO_4 (Pot. phosphate, dibasis)	2 gm.
3.	Agar	15 gm.
4.	Distilled water	1000 cc.

Prepare in the usual manner, adjust pH to 7.4-7.5, distribute in 1 liter flasks 600 cc. This is best base agar.

5.	Lactose 10 gm. of 20% sterile sol.	50 cc.
6.	Eosin, yellowish, 2% aqueous sol.	20 cc.
7.	Methylene blue, 0.325% aqueous sol.	20 cc.

Just before use, to each 100 cc. of the base agar.

Lactose, 20% sol., sterile	5 cc.
Eosin, yellowish, 2%	2 cc.
Methylene blue	2 cc.

Mix well and pour into Petri dishes.
Use: For the routine determination of organisms of the colonaerogenes groups in water.
Note: For the isolation of pathogenic organisms from feces, it is necessary to reduce the dye content one half. Sterilize the lactose 10 lbs. pressure ten minutes.

Russell's Double Sugar

To 100 cc. of beef extract agar pH 7.3 - 7.4, add: 1 gm. of lactose and 0.1 gm. glucose. This may be dissolved in a minimum of water. 5 cc. 0.02% aqueous solution phenol red. Sterilize in the autoclave at a pressure of 8 lbs. for 25 minutes. Slant with a deep butt. Upon solidifying check the reaction with known cultures of E. Coli, Para "B" and E. Typhosa; the stab and streak method being employed. The reactions are as follows:

E. Coli - - acid and gas throughout the whole tube.
Para "A" or "B" - acid and gas butt. Alkaline slant.
E. Typhosa - acid butt alkaline slant.

Sabourand's Medium
(for fungi)

Peptone	10 gm.
Maltose, crude	40 gm.
Agar	20 gm.
Distilled water	1000 cc.

Adjust reaction to pH 5.2; tube and slant; autoclave at 8 bls. for 30 minutes.

Blood Agar

To 100 cc's of Beef Extract agar (pH 7.2 - 7.4), melted and cooled to about 40°C. add: 5 cc's of sterile citrated blood. Mix and pour into either sterile test tubes (agar slants) or sterile petri dishes (blood plates). Incubate at 37°C. for 24 hours to insure sterility.

BACTERIOLOGICAL EXAMINATION OF WATER AND MILK

I. Water:

It is customary to submit specimens of drinking water from army stations for bacteriological examination at frequent intervals. If laboratory facilities are available, this is done locally; otherwise, it is shipped to the nearest Service Command Laboratory, or to the Army Medical School.

The purpose of a bacteriological examination of milk and water is to determine the potability. Generally, two different tests are done (1) to determine the total number of bacteria by means of the standard plate count; (2) to ascertain if there has been fecal contamination by demonstration of organisms of the Colon-Aerogenes Group.

A. Apparatus: all apparatus must be sterile.
   1. Glass stoppered 120 cc. sample bottles, protected by muslin cap.
   2. Pipettes 10 cc, 1 cc, with cotton plugs in the mouth ends.
   3. Glass test tubes for making dilutions.
   4. Petri dishes.

B. Culture Media.
   1. Final reaction for broth and Agar Media should be between pH 6.4 and 7.
   2. Following is a list of media used:
      a. Lactose broth.
      b. Nutrient Agar.
      c. Clark and Lub's Medium.
      d. Simmon's citrate agar slants.
      e. E.M.B. plates.
      f. Brilliant Green Bile Lactose 5% and 2%.
      g. Dulcitol.

C. Reagents.
   1. Sterile distilled or tap water.
   2. 0.04% solution of methyl red in 60% alcohol.
   3. 10% aqueous solution KOH.
   4. Xylol.
   5. 90% alcohol.
   6. Loeffler's Methylene Blue.
   7. Phenolphthalein indicator. 1% in 50% E. alcohol.
   8. Concentrated H_2SO_4.
   9. NaOH, N/10.

D. Special Apparatus.
   1. Babcock pipettes 17.6 cc.
   2. Babcock fat bottles.
   3. Lactometer with Quevenne scale.
   4. Wooden racks with holes for 5 large fermentation tubes and 2 small fermentation tubes.
   5. Lens magnifying 2 1/2 times for plate counts.
   6. Incubator with temperature set at 37.5°C.
   7. 56° Water Bath.
   8. Centrifuge.

E. Collection of Specimen.
   1. Must be done under as sterile conditions as possible.
   2. Should be representative.
   3. Collected in a sterile glass-stoppered bottle.
   4. If some time elapses between receipt of specimen and time tests are made, it should be placed in a refrigerator.

F. The Total Bacterial Count.
   1. Dilutions.
      a. Undiluted: 1:10; 1:100; 1:1000, etc.
      b. Set up two test tubes containing 9 cc. of sterile water; in the first tube and 1 cc. of undiluted water and in the second transfer 1 cc. of water from the first tube.
   2. Plating.
      a. Transfer 1 cc. amounts from the 1:10 dilution and the 1:100 dilution to two sterile Petri dishes.
      b. Add about 10 cc. of melted nutrient agar, cooled down to 40°C. to each Petri dish.
      c. Mix by tilting and rotation.
      d. Allow to solidify and incubate.
      e. Pour a plate of nothing but agar, and
      f. Pour a plate using 1 cc. of water which was used for the dilutions.
      g. These plates are controls.
   3. Incubation: plates should be incubated for 24 hours.
   4. Counting.
      a. Use a lens with a magnification of 2 1/2 times. A special ruled apparatus is made for plate counting.
      b. Try to prepare dilutions so that at least 2 plates will give from 30 to 300 colonies.
      c. Count the total number of colonies and multiply by the dilution factor. For example, if 140 colonies are counted on the plate where the 1:10 dilution was made, then 140 x 10 = 1400 colonies, which is the plate count.
      d. Under 500 colonies per cc. is considered potable raw water. Over 500 colonies, unpotable. 200 colonies per cc. or less is potable for treated water.

G. Tests for the presence of Coli-Aerogenes.
   1. Introduction and definition.
      a. Gram-Negative non-spore forming bacilli which ferment lactose with gas formation and grow aerobically on standard solid media.
      b. Formation of 10% gas or more in a standard lactose broth fermentation tubes within 24 hours at 37°C. is presumptive evidence of the presence of members of this group.
      c. Appearance of lactose-splitting colonies on E.M.B. plates made from a fermentation tube with gas confirms considerably the presumption that gas formation was due to the presence of members of Coli-Aerogenes Group.

d. To complete the demonstration of the presence of members of this group, it is necessary to show that one or more of the aerobic plate colonies are gram-negative non-spore-bearing bacilli, which, when inoculated into a lactose broth, fermentation tube, form gas.

2. Presumptive Test.
   a. Inoculation
      (1) At least twice as much media as water.
      (2) Inoculate 5 large fermentation tubes with 10 cc's water, two small fermentation tubes, 1 with 1 cc. and the other with 1 cc. of the 1:10 dilution mentioned before.
   b. Incubation and reading.
      (1) Incubate the tubes for 48 hours. Examine at 24 hours and 48 hours and record gas formation. Records should be such as to distinguish between:
         (a) Absence of gas formation.
         (b) Formation of less than 10% gas in the inverted tubes.
         (c) Formation of more than 10% in the small tubes.
   c. Positive Presumptive test: formation of 10% or more gas within 24 hours constitutes a positive presumptive test.
   d. Doubtful Test.
      (1) No gas or less than 10% within 24 hours.
      (2) The presence of gas in any amount at 48 hours.
   e. Negative Test: absence of gas formation after 48 hours.
3. Partially Confirmed Test.
   a. At the end of 48 hours, if gas has formed in tubes containing less of the water specimen, then plate. (For example, if water has been tested in amounts of 10 cc, 1 cc and 0.1 cc, and gas is formed in 10 cc and 1 cc, not in 0.1 cc, the test need be confirmed only in the 1 cc amount).
   b. Make transfers to plates as soon as possible after gas formation. If gas occurs at the end of 24 hours, then transfers may be made.
   c. Make one or more Endo or E.M.B. plates from the tube which shows gas formation from the smallest amount of water tested.
   d. Incubation of plates: incubate the plates for 18 to 24 hours.
   e. Results, typical and atypical:
      (1) If typical colonies have developed during the time of incubation, the partially confirmed may be considered positive.
      (2) If no typical colonies develop within 24 hours, incubate for another 24 hours.
      (3) If no typical colonies develop after 48 hours, then two or three colonies most likely to be Coli-Aerogenes, are chosen.

4. Completed Test.
   a. After the colonies are selected, either typical or atypical, they are inoculated into the following.
      (1) 2 tubes of Clark and Lub's.
      (2) 1 Simmon's Citrate Agar.
      (3) 1 dulcitol sugar fermentation tube.
      (4) Brilliant Green Bile Lactose, 5%, small tube.
   b. All incubated 24 hours to 36 hours, except Clark and Lub's.
   c. Following is the reaction obtained on the above mentioned media:
      (1) For Colon Group:

(a)	Voges-Proskauer Test-Neg.	
(b)	Methyl Red	Positive
(c)	Citrate	Negative
(d)	Dulcitol	Acid and Gas
(e)	Brilliant Green	Gas

      (2) Aerogenes Group.

(a)	V.P	Positive
(b)	M.R.	Negative
(c)	Citrate	Positive
(d)	Dulcitol	Negative
(e)	Brilliant Green	Gas

      Brilliant green bile lactose is used to rule out all organisms that ferment lactose, but are not of the Coli-Aerogenes Group.
   d. Procedure for Voges-Proskauer (V.P.) and Methyl Red (M.R.) Tests:
      (1) V.P. - to a Clark and Lub's tube, inoculated and incubated for 36 to 48 hours, add 5 cc. of 10% KOH (or the same amount of KOH as broth). The color develops slowly and a long time should elapse before reading. A pink fluorescence indicates a positive, no color, a negative reaction.
      (2) M.R. - add 5 drops of an 0.04% solution of Methyl Red in 60% alcohol to a Clark & Lub's tube, which has been inoculated and incubated 48 to 92 hours. A red color is positive and a yellow color negative.
   e. Reaction of Simmon's Citrate: Simmon's Citrate Agar slants are dark green and if positive, there is a dark blue slant.
5. Swimming Pool Water - water in swimming pools should be of the same standards as drinking water.
6. If colonies are found on the E.M.B. plate resembling organisms of the Typhoid Dysentery Group, Russell's Double Sugar tube should be inoculated.
7. Sewage: sewage and grossly polluted water may be examined the same as other water, but higher dilutions must be used.

II. Milk.

A. Enumeration of Bacteria

1. Standard Plate Count.

   a. Dilutions: dilutions are made as with water, 1:100, 1:1000 are usually used. The milk specimen should be shaken 25 times with an up and down motion of about 1 foot.

   b. Transfer 1 cc. amounts from each tube of diluted milk (sterile water is used), to a sterile Petri dish.

   c. Nutrient agar is used as in water; it should be cooled to 40 to 45°C.

   d. Incubate the plates at 37°C. for 48 hours.

   e. Colonies are counted and the number determined as in water analysis.

   f. Standard Plate counts may be made on buttermilk, cream, chocolate milk, raw milk, ice cream, etc.

2. Test for the presence of organisms of the Coli-Aerogenes Group.

   a. Inoculate three small fermentation tubes containing 2% Brilliant Green Bile Lactose with the following: Standard Plate (1:10 dil.); 2nd tube, 1 cc. of the 1:100 dilution; and the 3rd tube, 1 cc. of the 1:1000 dilution.

   b. Incubate for 48 hours.

   c. If gas is present, proceed with the partially confirmed and completed test as described under WATER G3 and 4).

3. Microscopic Count of Bacteria (Breed Method).

   a. Exactly 0.01 cc. of milk is drawn up into a special capillary pipette (M.S. item No. 43540) and is spread over an area of 1 sq. cm. on a microscopic slide.

   b. The uniformly spread film is dried in a warm place for not more than 5 to 10 minutes.

   c. (1) Immerse in Xylol one minute to remove fat. Drain and allow to dry.

      (2) Fix for one minute in 90% alcohol.

      (3) Stain with Loeffler's methylene-blue solution.

      (4) Rinse with water.

      (5) Decolorize with alcohol until only a faint blue tint is left.

      (6) Dry and examine microscopically.

   d. The number of bacteria per cc. of milk is estimated by counting all the organisms within a given area in a microscopic field, this area having been carefully measured and its ratio to a square centimeter determined. At least 1/100,000 part of a cc. of milk is to be examined. The microscope should be so adjusted that each field covers a certain known fraction of the area of a square centimeter.

e. Advantages.
   (1) Makes possible the counting of all bacteria living and dead, and can therefore be used on specimens preserved with formalin or other antiseptics.
   (2) It is more economical and can be carried out rapidly in the field.
   (3) It gives information concerning the sanitary condition of the dairy and the contaminated milk before pasteurization.

f. Disadvantages.
   (1) The small amounts of milk used lead to inaccuracy and the large factors used in estimating the bacterial count introduce a large factor of error.
   (2 Much time must be spent on the counts, in order to reduce this factor of error, especially when examining very good milk.
   (3) The individual technic of the counter may be responsible for a greater variation in results than when the plate method is used.

g. The ratic used in comparing the standard plate count with the Breed Count is estimated at 1 to 4.

h. For preserving milk for shipment, use 1 cc. of formalin (37%) full strength, to 120 cc. of milk.

4. Methylene Blue Reduction Method: known as the reductive test. It is useful where laboratory facilities are limited, or making a rapid inspection of a large number or samples. It is based on the fact that when methylene blue is added to milk, the color may be reduced or lost, depending on the oxygen consumption of the bacteria present.

   a. Methylene Blue Reagent.
      (1) Standard tablet available manufactured by National Aniline Company.
      (2) Dissolve 1 tablet in 50 cc. of boiling distilled water and add 150 cc. of cold distilled water.

b. Technique.
   (1) Measure 10 cc. of milk in a thick-walled test tube fitted with a rubber or cork stopper.
   (2) Add 1 cc. of the certified methylene blue solution.
   (3) If the blue color is not evenly distributed, invert and mix uniformly.
   (4) Place in a water bath and heat to 37°C. This temperature is maintained until test is completed.
   (5) Tubes should be observed at 15 minute intervals and the end point recorded (disappearance of the blue color).

c. Interpretation of Results.
   Class 1 - Good milk, not decolorized in 5 1/2 hours.
   Class 2 - Milk of fair average quality, decolorized in less than 5 1/2 hours, but not less than 2 hours.
   Class 3 - Unsatisfactory milk, decolorized in less than two hours, but not less than 20 minutes.
   Class 4 - very unsatisfactory milk, decolorized in 20 minutes or less.

B. Tests other than Bacteriological.
   1. Specific Gravity.
      a. Ranges between 1.027 and 1.035.
      b. May be determined by means of a hydrometer. The Quevenne lactometer with a range of 1.015 - 1.040 is usually used, however, the hydrometer designed for taking the Sp.Gr. of urine may be used.
      c. Scale of Quevenne lactometer reads in two figures, which are the second and third decimals of the full sp. gr. reading, e.g., a milk of sp. gr. 1.030 would have a Quevenne reading of 30.
      d. Temperature should be 15.6 C. - corrections for readings close to this temperature are made by adding 0.0002 to the observed sp.gr. for each degree above and subtracting 0.0002 from the sp.gr. observed for each degree below 15.6° C. Such corrections should be made within a range of 13 to 18° C.
   2. Percentage of Fat - may run from 2.00% to 4.45%.
      a. Babcock Method.
         (1) Apparatus and Reagents.
            (a) Test bottles (Babcock)
            (b) Centrifuge - speed 600 - 1200 r.p.m.
            (c) Pipettes 17.6 cc and 17.5 cc graduations only.
            (d) Sulfuric Acid in commercial or technical, with specific gravity 1.82 to 1.83 at 20°C.
         (2) Procedure.
            (a) Transfer 17.6 cc of milk to the test bottle and 17.5 cc. of sulfuric acid, preferably not all at one time, pouring it down to bottleneck so as to wash down all traces of milk. Temperature of the acid should be between 15 and 20°C. Shake until all trades of curd disappear; then

(b) Transfer bottle to centrifuge, counterbalance it and whirl for 5 minutes after the proper speed is attained.

(c) Add soft or distilled water at 60°C., or above, until the bulk of the bottle is filled.

(d) Centrifuge 2 minutes.

(e) Add hot water until the liquid approaches the top graduation on the scale.

(f) Centrifuge one minute.

(g) Place bottle in a 55 to 60°C. water bath. Immerse it to the level of the top of the fat column. Leave in water bath 10-15 minutes.

(h) Remove bottle and with the aid of calipers or dividers, measure the column of fat from the lowest surface to the highest point of the meniscus.

(i) Fat column at time of measurement should be translucent, golden yellow or amber color, and free from visible suspended particles.

LABORATORY TECHNICIANS MANUAL

PART III

INDEX

Chapter | Page

CHEMISTRY

1. Scope and definitions - Chemistry is a branch of natural science. It is, in fact, a branch of physics, which is the science dealing with changes in matter, with the composition of matter, and with the properties of matter.

a. Physics deals with changes in the state of matter, or with the properties of matter in large masses or general aspect. It is also concerned with changes in matter which do not involve molecular rearrangement or composition.

b. Chemistry deals with two main groups of phenomena, namely:

(1) The composition of substances, especially the structure of their molecules, and the properties which are dependent upon this structure.

(2) Changes in the composition of matter or of molecular structure and the effects attending these changes.

c. For example, suppose we consider a block of wood which is placed in an oven and heated. After a period, the block of wood becomes hot or heated. Thus far the change is a physical one. When, however, the heat is increased and prolonged the block of wood burns and finally no wood is left. What we call "ash" and "smoke" have been produced from the wood. Chemistry is here involved and the change, burning, is a chemical change.

d. General Chemistry (Theoretical Chemistry or Physical Chemistry) treats with principles which generalize the facts relating to the composition of substances and to changes in composition. In other words, it deals with the fundamentals underlying all chemistry and chemical action. It is the branch of chemistry we shall study first.

2. Matter and its subdivisions - In the study of any scientific subject, it is essential to define one's terms in order that all who work with them are "speaking the same language." Scientific definitions will be found, usually, to be limitations upon the meanings of words, making them more specific than the ordinary or general meaning of the word used in everyday speech or writing. We will proceed to define matter and some of the terms used to describe it, its divisions and its properties.

a. Matter is anything which has weight or occupies space.

b. A substance in chemistry, is a particular kind of matter.

(1) A pure substance is a substance which when divided yields particles of identical properties with each other and with the original substance.

c. A property of matter is a means or any means by which matter makes itself evident to our senses. All of our knowledge of matter has been gained by observation of the properties of matter. Examples are given below under general and specific properties.

(1) General properties of matter are those possessed by all matter regardless of kind. Most of these are well-known to us, so well known that we take them for granted and do not often think about them. For instance, we know that matter cannot be destroyed, we know that two bodies cannot occupy the same space at the same time. In other words, matter has the general properties: indestructibility (it cannot be destroyed) and impenetrability (no two bodies can occupy the same space at the same time).

(2) Specific properties of matter are peculiar to each kind of matter and are usually described by the degree or extent to which they are possessed by a substance. It is upon these properties we base our work in chemistry. In fact, we recognize substances by means of properties which they possess to some marked degree. For some examples: solids have the properties of hardness, form, solubility, melting point, density and others. Liquids have density, boiling points, color, odor, taste and other properties. Gases have color, odor, taste, critical temperature, density and many other properties.

(a) Specific properties of any substance are usually measurable; that is we have some means of determining the degree to which a substance has a property.

(b) Specific properties of any substance are always possessed by that substance to the same degree, if other conditions are held constant. In other words, ice always melts at $0^{o}C.$; boric acid always dissolves in water in the proportion of 1 Gm. of boric acid to 18 cc. of water; mercury always weighs 13.6 Gm. per cc. under the same conditions.

d. Mass or weight - Mass or weight will be treated in our work as identical terms. They refer to the quantity of matter. Weight is defined as the measure of the earth's attraction for a body. In actual practice the attraction of the earth for a body is compared with the same attraction for a standard body (a weight), - a gram, or a pound, etc.

(1) A <u>body</u> is any particle of matter which can make its presence known to any or all of the five senses of man.

(2) A <u>molecule</u> is the smallest particle of matter which is capable of independent existence. It is, for each substance, definite in size, weight and composition. These sizes and weights will be considered at a later date. The whole of our course will be concerned in a large part with the composition of molecules.

(3) An <u>atom</u> is a particle which with one or more other atoms goes to make up a molecule. It is then a division of a molecule but it is <u>not</u> capable of independent existence. All atoms of a given element are alike and are uniform in composition and weight.

(4) <u>Ions</u> are atoms endowed with a charge of electricity and temporarily combined with a solvent (usually water). They are not existing independently, although they have become dissociated from the molecule to which they previously (before being dissolved) belonged. They will be discussed in some detail at a later date.

(5) Smaller divisions of matter, electrons, protons, etc., will not be considered in this course.

The <u>condition</u> or <u>state</u> of matter refers to matter as it is affected by the circumstances which surround it. For instance, we say that a body is hot or cold, or under pressure. Here frequently, however, we refer to the states of matter known as solid, liquid and gaseous.

(1) <u>Solid Matter or matter in the solid state</u>, means above and beyond our ordinary conception of the term, that the molecules of solid matter are fixed in relation to one another; that is, they cannot move around or away from one another. This, of course, explains why solids have their fixed form.

(2) <u>Liquid matter, or matter in the liquid state</u>, is matter whose molecules have sufficient freedom of motion to move around each other, but yet have sufficient attraction one for another to maintain a relatively constant bulk or volume. This makes liquids capable of assuming the shapes of the vessels used to contain them.

(3) <u>Gases or matter in the gaseous state</u> is matter whose molecules have complete freedom of motion and in addition a certain repulsion for each other. This is what causes the gases to tend to expand or exert pressure on their containers.

3. <u>Energy</u> - Modern day physics treats energy separately from matter. We will continue under the older idea of considering energy to be a property of matter; namely, its capacity to do work. This is much simpler and for all the use we will make of it, as adequate as the modern conception of energy as something fundamentally similar to matter and ultimately interchangeable with it.

4. <u>The Laws of Nature or Natural Laws</u> - One of the first ideas that the beginner in any science must learn is that natural laws were made <u>after</u> the lawmakers had observed the acts of nature. There is no way to make nature obey these "laws." With this fact as a background, we can define a Law of Nature as a statement of someone's experience; and the collection of assembled fact commonly referred to as Natural Laws may be defined as the combined experience of the years which have been spent studying the various sciences. They merely state that, given such and such a state of conditions, this and that action will result. Most natural laws are experiences which have been repeatedly undergone, without ever having been disproven. In many cases they are capable of application as mathematical formulas. Not all of these "laws" apply with such accuracy, however, and according to their accuracy, laws are classified as EXACT LAWS, APPROXIMATE LAWS, AND EMPIRIC LAWS.

a. Most of the "laws of nature" began as theories or hypotheses in the mind of some investigator. These theories are merely plausible explanations or suppositions advanced in an attempt to explain certain observed facts. In other words, the investigator says to himself, "This is how and why it <u>might</u> have happened." He then has a theory. Next he says, "If that is how and why what I saw happened, then if I do thus and so, then this is what is going to happen." He tries it, and if what he expected does happen, the investigator has a hypothesis. These theories and hypotheses are used as guides to show the direction further experiment should take, or perhaps, to point the way to direct proof in some manner or other. If such proof is accomplished, then the theory or hypothesis becomes a "law."

b. <u>Exact Laws</u> - Exact Laws are those to which no exceptions are known; and any minor deviations in the results of application of such laws which occur, become increasingly small as the skill of the manipulator increases.

(1) The Law of the Conservation of Matter is such a law. It states that matter cannot be destroyed. In other words, regardless of what changes matter may be made to undergo, its weight remains the same, none being destroyed, none being created. This is one of the oldest as well as one of the most exact of all natural laws. It has been expressed in many ways, down through all time. We are all familiar with the old proverb, "From

nothing, nothing comes." In chemistry, a favorite statement of the law is, "You can't get anything out of a test tube that you didn't put into it." As a corollary of this law, we see that the total of all the matter in the universe is and will always be a constant.

c. Approximate Laws - Approximate laws of nature are those which are true for most conditions, but vary under some few conditions (usually at some extreme). Minor variations in the results of application of approximate laws usually remain the same regardless of the skill of the investigator who is applying them.

(1) Boyle's Law is an example of an approximate law of nature. It states that the volume of a gas varies in inverse proportion to the pressure exerted on the gas. This works out according to $p : p' :: v' : v$, with mathematical precision for ordinary temperatures and pressures. Near the point of liquefaction of gases, that is to say, at very low temperatures, and very high pressures, it fails to describe what happens. Most approximate laws are eventually corrected by further study which discovers some minor fact previously not taken into consideration. Approximate laws also have usually some easily seen basis in fact, that is, they explain what happens.

d. Empiric Laws - Empiric Laws are merely statements of observed facts with no attempt whatever to explain anything. They are frequently only gross approximations and may even vary widely from fact. On the other hand, they are often convenient for arriving at some result not easily seen or understood. Unlike either exact or approximate laws or even well founded theory or hypothesis, they seldom can be made of use as a guide to further study.

(1) Trouton's Rule is an example of an empiric law. With no attempt at an explanation, it states that it takes 22 times the number of calories expressed by the boiling point of a liquid to boil or volatilize a gram-molecular weight of the liquid.

5. Atomic and Kinetic Theory - We are now ready to begin the study of chemistry. Our approach is perhaps not the usual one for we will omit historical and other considerations and begin with the ultimate particle or piece of matter and build up our picture of what happens from there. Moreover, we have not time in the short course allotted us to attempt a great deal of proof either in the laboratory or mathematically and theoretically on paper. We will have to accept as fact, and it is such, the existence and behavior of these particles or units of matter.

a. Almost ever since men began to try to puzzle out why things happened under certain circumstances, it has seemed logical that matter was composed of an enormous number of small particles. As investigators became more skillful, it became more and more evident that this was the case, until the present day, and then only very recently has this been substantiated, the particles being observed with the new electronic microscope.

b. Atomic theory - in 1804 John Dalton in England formulated and advanced a theory built upon the lines suggested by the idea of such a structure of matter. From it he and others were able to reason ways in which the theory could be tested out. Not one of these deductions based on sound reasoning from Dalton's theory has yet failed to prove true. This atomic theory states that:

(1) Elements are made up of <u>inconceivably</u> (?) <u>small particles</u> which are <u>indivisible</u> in chemical actions and which are called <u>atoms</u>.

(2) The atoms not only have <u>definite weights</u>, but the <u>atoms of any one element have the same weight</u>, which is different from the weight of the atoms of some other element.

(3) When elements unite chemically, the <u>action takes place between the atoms</u>.

These three statements are really one of the basic principles of chemistry, and if the student will learn them well and think of them in connection with later work he will see that much useless rote learning will be avoided. For our purposes the statements may be regarded as undisputed facts and the student is required to learn them verbatim as facts.

c. <u>Kinetic theory</u> - Before and since the time that chemists were elaborating and proving experimentally that assumptions made upon the atomic theory were correct, physicists were developing a somewhat similar theory to explain certain phases of physics. They, of course, were interested in the larger particles which atoms made up when they united. Finally a sort of theory grew from their efforts, and it is still growing. Unlike the atomic theory, the molecular or kinetic theory was not the work of any one person and it is perhaps not so clearly stated; in fact, the theory cannot yet be completely stated because we are still building up proofs of it, and developing still further applications of its postulates. For our use, we will accept as established fact the following as the kinetic theory:

(1) Matter is composed of infinitesimally small particles called molecules.

(2) These molecules of any particular substance are indivisible by physical means, but can be divided into their constituent atoms by chemical means.

(3) The molecules of any particular substance are all alike and have a definite weight, this weight being known as the molecular weight of the substance.

(4) Each molecule of each substance is endowed with a certain measurable quota or quantum of energy. This quantity of energy is the same for all molecules regardless of the size or kind, being dependent only upon the temperature.

(5) By means of its endowed energy each molecule is in a state of rapid motion or momentum (depending on the temperature and state of the substance).

(6) A gram-molecular weight of any substance contains a definite number of molecules, and this number is the same for all substances. This number of molecules is 606,000,000,000,000,000,000,000, or better 6.06×10^{23}, and is called Avogadre's number because he was the first to use, not the number, but the basic principle.

Upon these six postulates which are the more fundamental principles of the kinetic theory, it is possible to explain or to predict so many of the laws and discoveries of science that chemists and physicists universally now accept the theory as proven.

It is easy to explain the behavior of matter in all of its forms by kinetic theory, and to deduce many facts that were well known before the theory assumed such large stature, as well as many facts that have since come to light as a result of deductions made from the theory. Let us consider the three states of matter previously discussed in paragraph 2 above.

Solids are definite in volume, external conditions being kept the same, and molecular motion is confined to rapid vibration of the molecules within the solid, without exerting enough pressure to change the external shell. Evidences of this vibration are the conductance of heat and sound through solids, electrical charge, and if it is a magnetic substance, the ability to maintain its magnetic polarity during division.

Let us now suppose this solid to be heated, thus adding to the energy of its molecules, for heat is a form of energy. When sufficient heat has been added, the substances

lose its form because the molecules have acquired sufficient energy to move around one another. We say that the substance has melted. Note that the molecules of the substance attract each other for the most part and make no attempt to repel each other; a few molecules, however, do acquire enough energy to escape through the surface shell of molecules, and the higher the temperature the more the energy and the greater the number that will so escape. We say that the substance has evaporated and we know that evaporation takes place to a greater extent on a warm than on a cold day.

Suppose, now, we continue to heat the substance until the molecules become very violent, and break up the surface shell becoming gaseous or vapor molecules. Due to the great number, the great speed, and the loss of the power to cohere, the molecules are in a state of chaotic collisions, the molecules striking each other and the sides of the container and bouncing off in every conceivable direction. We say that the gas is exerting pressure on the sides of the container, just as a very hard shower of rain seems to be pressing on all points of a tent at one time.

The student is referred to pages 18 to 48 in Simons' Manual of Chemistry, for examples of phenomena dependent upon molecular motion and energy, and to pages 110 to 113 for a fuller discussion of atomic theory. These nine postulates are <u>very important</u> to a quick understanding of chemistry, which is the reason they have been introduced early in the course and they <u>must be learned</u>.

6. <u>Elements</u> - the word element means, fundamentally, something which cannot be changed. We use the term element in chemistry to mean a substance whose <u>molecule cannot be broken down into more than one kind of atom</u>.

a. <u>The number of elements</u> - eighty-nine elements are today well known to chemistry, and there is reason to believe that the total number of elements is limited to ninety-two. Of these 89 elements, not all are of importance to medicine and so to pharmacy, although such is the progress of medical science that they may become so at any time. For instance, helium once believed to be one of the most inert substances known is now being used in the treatment of asthma. There are, nevertheless, comparatively few of the elements that we need study in any detail. The elements are listed below more or less in the order in which we will study them. The underlined ones are those important to us in medicine:

As of 1943

(1) Gaseous elements - (of which there are eleven)

Hydrogen	H	1.008	1	
Oxygen	O	16.000	2,5	
Nitrogen	N	14.008	3,5	
Fluorine	F	19.000	1	
Chlorine	Cl	35.457	1	

Helium	He	4.002	0)	
Neon	Ne	20.2	0)	These are the so-call-
Argon	A	39.9	0)	ed "noble gases,"
Krypton	Kr	82.9	0)	they do not enter into
Xenon	Xe	130.2	0)	combination with any
Radon	Rn	222.	0)	other substance, so we
				are not concerned with
				them.

(2) Liquid elements - (of which there are only two)

Bromine	Br	79.916	1,3,5,7
Mercury	Hg	200.61	1,2

(3) Solid elements (of which there are 76). The following list is for reference and is inserted here only for convenience.

Lithium	Li	6.940	1)	
Sodium	Na	22.997	1)	The "ALKALI METALS";
Potassium	K	39.096	1)	All are metallic
Rubidium	Rb	85.44	1)	elements
Cesium	Cs	132.91	1)	

Copper	Cu	63.57	1,2)	
Silver	Ag	107.880	1)	All are metals
Gold	Au	197.2	1,2)	

Calcium	Ca	40.07	2)	
Strontium	Sr	87.63	2)	Metals
Barium	Ba	137.37	2)	
Radium	Ra	225.95	2)	
Beryllium	Be	9.02	2)	
Magnesium	Mg	24.32	2)	Metals
Zinc	Zn	65.38	2)	
Cadmium	Cd	112.41	2)	
Mercury	Hg	200.61	1,2)	
Platinum	Pl	195.23		

Scandium	Sc	45.10	3)	Metals
Yttrium	Y	88.9	3)	
Lanthanum	La	138.92	3)	
Actinium	Ac	227.	3)	
Boron	B	10.82	3)	All metals except
Aluminum	Al	26.97	3)	boron
Gallium	Ga	69.72	3)	
Indium	In	114.80	3)	
Thallium	Tl	204.39	3)	
Carbon	C	12.00	4)	Carbon and silicon
Silicon	Si	28.06	4)	are non-metals; the
Germanium	Ge	72.60	4)	others are metals.
Tin	Sn	118.70	2,4)	
Lead	Pb	207.2	2,4)	
Titanium	Ti	48.10	3,4)	Metals
Zirconium	Zr	91.00	4)	
Cerium	Ce	140.13	3,4)	
Thorium	Th	232.15	4)	
Nitrogen	N	14.008	3,5)	Nitrogen and Phos-
Phosphorus	P	31.02	3,5)	phorus are non-
Arsenic	As	74.96	3,5)	metals; others are
Antimony	Sb	121.77	3,5)	metals.
Bismuth	Bi	209.00	3,5)	
Vanadium	V	50.96	3,5)	Metals
Columbium	Cb	93.10	3,5)	
Tantalum	Ta	181.50	5)	
Protoactinium	Pa	231.00	)	
Oxygen	O	16.00	2)	Oxygen and sulfur
Sulfur	S	32.064	2,4,6)	are non-metals;
Selenium	Se	79.20	2,4,6)	others are metals
Tellurium	Te	127.51	2,4,6)	
Polonium	Po	210.0	)	
Chromium	Cr	52.01	2,3,6)	Metals
Molybdenum	Mo	96.00	3,4,6)	
Tungsten	W	184.00	6)	
Uranium	U	238.17	4,6)	
Fluorine	F	19.00	1)	Non-metals
Chlorine	Cl	35.457	1,3,5,7)	The Halogens
Bromine	Br	79.916	1,3,5,7)	
Iodine	I	126.932	1,3,5,7)	
Manganese	Mn	54.93	2,3,4,6,7)	Metals
Masurium	Ma	98.00		
Rhenium	Re	186.31		

Iron	Fe	55.84	2,3	) Metals
Cobalt	Co	58.94	2,3	)
Nickel	Ni	58.69	2,3	)
Ruthenium	Ru	101.7	3,4,6,8	) Metals
Rhodium	Rh	102.91	3	)
Palladium	Pd	106.7	2,4	)
Osmium	Os	190.8	2,3,4,8	)
Iridium	Ir	193.1	3,4	)
Platinum	Pt	195.23	2,4	)
Hafnium	Hf	180.8		)

It will have been noted that the list above contains metals, non-metals, gases, liquid, and solid elements.

b. Elementary molecules - Most elementary molecules consist of two identical atoms which are united chemically to form the molecule. Thus most of the molecules of elements weigh twice as much, proportionately, as their respective atoms. A few monatomic elements are known, and a few whose molecules contain more than one atom. In every elementary molecule, however, all of the molecules are alike.

c. Families of elements - The student will find in his reading, references to "families of elements." It is not intended to go deeply into a study of elementary families at this time; and it is sufficient to point out that the elements are grouped into families according to the similarity of their more important properties. In the list of elements given under (3) above, the elements are grouped according to families, and reference will be made to the similarity of these properties from time to time.

7. Compounds - (Text Reference, Simons, page 56). We defined an element, in the previous paragraph as a substance whose molecules contain only one kind of atom. In contradistinction, a compound is a substance whose molecules contain more than one kind of atom; and a compound may be broken down chemically into two or more different kinds of atoms. The idea of the term compound also implies that the two or more atoms are combined chemically, which is to say, they are held together by a force, which is called chemical affinity, or chemism.

a. Examples:

Salt or sodium chloride contains in each of its molecules:

1 atom of chlorine
1 atom of sodium

Calcium Carbonate or chalk contains in each of its molecules:

1 atom of calcium
1 atom of carbon
3 atoms of oxygen

b. LAW OF CONSTANCY OF COMPOSITION and LAW OF MULTIPLE PROPORTIONS - Several of the basic laws of chemistry are intended to help one to understand the formation of compounds. Two which have a direct bearing on the subject are the laws of (1) constancy of composition, and (2) multiple proportions.

(1) The LAW OF CONSTANCY OF COMPOSITION states that: A definite chemical compound always contains the same elements in the same proportion.

(2) The LAW OF MULTIPLE PROPORTIONS states that: If two elements, "A" and "B" are capable of uniting chemically in more than one proportion, then the quantities of "B" which can combine with a fixed quantity of "A" bear a simple ratio to one another. (By a simple ratio, here, we mean a ratio between <u>small whole numbers</u> like 1 to 2 or 2 to 3).

(a) Examples: there are two chlorides of iron as follows:

Ferrous chloride contains:	Ferric chloride contains:
1 atom of iron	1 atom of iron
2 atoms of chlorine	3 atoms of chlorine

Likewise, there are two oxides of hydrogen:

Hydrogen monoxide contains:	Hydrogen dioxide contains:
1 atom of oxygen	2 atoms of oxygen
2 atoms of hydrogen	2 atoms of hydrogen

8. <u>Symbols</u> - In writing about chemistry and chemicals, it was early found that the names we give substances, and the manner of stating what they contained, were cumbersome to handle. The examples shown in the two previous paragraphs are ample proof of this difficulty. A method of indicating more easily what was meant was needed to save words and space and to indicate the intention of the writer more clearly. Even the ancients recognized the need and their writings contain crude symbols or sign writing. Accordingly, a system of shorthand writing was developed. <u>Symbols</u> were developed for each <u>element</u>, and out of these symbols, it is possible to write <u>formulas</u> for each <u>compound</u>. Later, ways were learned to write the manner in which compounds and element entered chemical <u>reactions</u>, and these are called <u>chemical equations.</u>

a. What a symbol is:

(1) A symbol usually consists of the initial letter of the Latin name of the element. Where two or more elements begin with the same initial letter, some other distinctive letter from the name of the element is written beside the initial letter. The initial letter is written as a capital, the second letter as a lower case letter.

(a) Examples of symbols -

Single letter symbols:			Two letter symbols:		
Argon	-	A	Aluminum	-	Al
			Arsenic	-	As
Boron	-	B	Bismuth	-	Bi
			Bromine	-	Br
Carbon	-	C	Calcium	-	Ca
			Chromium	-	Cr

(2) A symbol means one atom of an element - In addition to being merely an abbreviation for the name of an element, a symbol refers to a single atom of an element. Thus, A above means 1 atom of Argon; Al means 1 atom of aluminum, Ca means 1 atom of calcium, etc.

(3) A symbol means a definite relative weight of an element. In addition to the above meaning, we may read into each symbol, in addition to the name and number of atoms, a certain definite relative weight of an element, as contemplated in the statement of atomic theory. This, then, is a complete statement of what a symbol tells us:

A means an atom of argon having a definite weight;
As means 1 atom of arsenic having a definite weight;
Cr means 1 atom of chromium having a certain weight, etc.

In the list of elements in paragraph 6 above, the symbols are written as the second column, directly following the name of each element.

(4) Subscripts - where it is desired to write a symbol which is to indicate more than one atom of an element, a subscript is used. A subscript is a number written to the right of a symbol to indicate the number of atoms or atomic weights desired. Examples follow:

As_2 means two atoms of arsenic or two atomic weights of it.

C means an atom of carbon or 1 atomic weight.

H_{35} means 35 atoms of hydrogen or 35 atomic portions of it by weight.

9. Atomic Weight - the atomic weight of an element is the relation between the weight of 1 of its atoms and an atom of oxygen. It is necessary to elaborate a little on what the above statement means.

An atom of any element is by definition, "an inconceivably small" particle. If it is inconceivable, then it is certainly too small to weigh. Recently we have learned the number of atoms into which a certain weight of substance can be divided, and we can calculate the weight of an atom. Such a weight, however, runs to upwards of 20 decimal places and is hard to apply to any practical use. Rather early in the study of chemistry it was learned that the relative weights of elements could be determined. We could determine that oxygen weighed 16 times as much as hydrogen per unit volume, and Avogadro early proved that, other things being equal, equal volumes of elementary gases contain the same number of molecules. From that, it was concluded that an atom of oxygen weighed 16 times as much as an atom of hydrogen. And in a similar manner, these relative weights were obtained for other elements. Instead of using the long statement that "oxygen weighs 16 times as much per atom as hydrogen," we say that the atomic weight of oxygen is 16, or simpler still, O = 16.

In the list of elements in paragraph 6a above, the figures in the third column are the atomic weights corresponding to the elements whose names and symbols occupy the first two columns. It will be noted that no units of weight are given. This, of course, is because the weights shown are relative weights, or ratios between the elements named and a standard or reference element.

Hydrogen, because it is the lightest known element, was, at first, chosen for the standard by which the weights of other elements were compared. The weights of other elements were determined by weighing the amount of another element which had combined with hydrogen. If it was found that, for instance, 35 parts by weight of chlorine, had combined with 1 part by weight of hydrogen, then the atomic weight of chlorine was called 35. In similar manner, the weights of other elements were determined and, in addition, other ways of comparing the weights were devised. With progress the atomic weights became more and more accurate, the elements under study became more and more numerous, and the number of known atomic weights increased. It was found that relatively few of the atoms combined directly with hydrogen, and that in a great many cases, awkward decimal fractions were obtained. Some investigators began to compare the other elements to oxygen because more elements combine directly with that element, and found that the weights so obtained were nearer to whole numbers. Gradually the use of oxygen as a standard became established and is universal today. SO THE STANDARD FOR ATOMIC WEIGHTS IS THE RELATION O = 16, instead of the older H = 1.

It will be seen that for rough work there is very little difference (if O = 16, then H = 1.008); but for fine work the oxygen standard is preferred, and all tables of atomic weight are so made.

Use of symbols and atomic weights - the student might well ask at this time, "How many atomic weights and symbols am I expected to learn?" The answer is, "None." It is common practice to use tables in order to obtain this data, and in our work access to tables will be allowed or necessary data will be furnished in all work. It will be found as a matter of practice, that symbols are so handy and so useful that they teach themselves to the student, and he very quickly begins to use them to save time. It is not necessary to spend time actually memorizing a set of symbols.

10. Formulas - (Text reference page 113) - (Simons). Formulas bear the same relationship to compounds that symbols bear to elements. They are shorthand representations of what a compound contains or is composed of. They are simply a concise statement of the number of and kind of atoms in a molecule of the compound. Also, in an analogous manner to the writing and reading of symbols, we mean by the formula for a compound, 1 molecule of the compound, weighing a certain definite relative amount. (This molecular weight is the sum of the weights of the atoms which the molecule contains).

a. Examples of formulas - we have used in our discussion thus far, several compounds as examples in various instances. These are tabulated below, with their formulas, for comparison to the cumbersom method previously used to write their composition:

Sodium Chloride	-	$NaCl$
Calcium Carbonate	-	$CaCO_3$
Ferrous Chloride	-	$FeCl_2$
Ferric Chloride	-	$FeCl_3$
Hydrogen Monoxide	-	H_2O
Hydrogen Dioxide	-	H_2O_2

b. The writing of formulas - Formulas are written according to certain customs which have grown up, and are well understood by chemists everywhere. Some of these follow:

(1) Write formulas with the metallic elements on the left and other elements following.

(2) Formulas are read from left to right.

(3) Subscripts are used to indicate the number of atoms of each element in the compound.

(4) Coefficients (numbers placed before the whole formula and on a line with it) are used to indicate the number of molecules intended; e.g., 2 NaCl means two molecules of sodium chloride, and therefore, two atoms of sodium and two atoms of chlorine, 3 $CaCO_3$ means three molecules of calcium carbonate and, therefore, 3 atoms of calcium, 3 atoms of carbon, and 9 atoms of oxygen. This is important in many respects. From a standpoint of weight alone, there would be no difference between 2 NaCl and Na_2Cl_2. From the standpoint of molecular make-up, however, 2 NaCl means two molecules each of which contains two atoms, whereas Na_2Cl_2 means one molecule which contains four atoms. This must be kept in mind. It is also necessary to remember that a coefficient multiplies every atom in a formula which it precedes, while a subscript multiplies only that atom to which it is attached (follows).

c. It is necessary that a compound have a definite composition before a formula can be written for it; but this follows from the law of constancy of composition and our definition of a compound.

11. Combining power of the elements - we have already stated that a force, chemical affinity, holds compounds together, and some of you may have begun to wonder why one molecule has only two atoms, another three, a third fifty-six, etc. The reason is that not all elements have the same amount of combining power. Some unite with others in a one to one ratio, while the same element will react with another different element in a 1 to 2 ratio, or some different ratio altogether. That phase of an element's combining power which determines its power to hold 1 or 2 or 3 other atoms in combination is called valency or atomicity, valency is the commoner term.

Valence a measure of combining power - Valency is one of the measures of combining power; it is not the force itself. Valence tells us how many atoms of another kind an atom will combine with. Naturally, some basic measure is necessary, some unit of measure, by which we may express the "size" or amount of combining power. Again hydrogen is taken as a basis; and we say that any element which combines with hydrogen in the proportion of 1 atom per atom of hydrogen has a valence of one, and that any element which combines with hydrogen in the proportion of 2 atoms of hydrogen atom of itself has a valence of two, and so on. Thus chlorine combines with hydrogen in a 1 to 1 ratio, forming hydrogen chloride - HCl, so we say that chlorine has a valence of one. Likewise, sulfur combines with hydrogen in a 1 to 2 ratio, forming hydrogen sulfide - H_2S, so we say that sulfur has a valence of two. Nitrogen combines with hydrogen in a 1 to 3 ratio, forming ammonia - NH_3, so we say that nitrogen has a valence of three, etc.

12. Structural Formulas - By means of the valence bonds mentioned in the previous paragraph, we can draw a diagram of a molecule of a compound instead of merely writing its formula. Such diagrams are called structural formulas. They are merely an indication of how we believe the elements of a compound to be united. They are not to be thought of as actual pictures of compounds, although they are almost as useful as such pictures would be if we had such things. Examples of such structural formulas are shown below, along with the name and molecular formulas of the compounds:

Hydrochloric Acid	Potassium Iodide	Carbon Monoxide	Carbon Dioxide
HCl	KI	CO	CO_2
H - Cl	K - I	C = O	O = C = O

Ferrous Chloride	Ferric Chloride	Calcium Carbonate	Water
$FeCl_2$	$FeCl_3$	$CaCO_3$	H_2O
Cl - Fe - Cl	Cl - Fe - Cl	O - C = O	H - O - H
	Cl	Ca- O	

13. <u>Radicals</u> - A great many chemical compounds contain groups of elements which have a character of their own and which, in reactions, move from compound to compound as a group, seemingly without internal change. Such groups of elements are called radicals. These radicals are well known in organic reactions, so well known that many of them have acquired names: we speak of the hydroxyl radical, the nitrate radical, the sulfate radical, the phosphate radical, etc. Radicals act as though they were single elements in reactions, moving intact from salt to salt. Radicals also are said to have valence, which is probably a misnomer; but if we draw structural formulas of various radicals we see that there must of necessity remain certain free bonds attached to some of the elements of the radical. It is these free bonds which give radicals their valence. Some typical radicals are shown below:

Hydroxyl Radical	Sulfate Radical	Nitrate Radical	Phosphate Radical	Ammonium Radical
-OH	SO_4	NO_3	PO_4	NH_4
	O	O	O	H
	O S O	O N	O P O	H N H
	O	O	O	H

14. Acids - Inorganic chemicals may be divided into several groups, each of which has characteristics common to all of the chemicals in the groups. One of these groups is the one containing what we call acids. We call all of them acids because they have certain properties in common and we call these properties "acid properties."

a. Common properties of all acids:

(1) Structure - all acids are compounds of hydrogen with some other element, a non-metal. We say that they have the general formula, HR or H^+R^-, where H or H^+ represents hydrogen, and R or R^- represents another element or radical containing another element. The plus and minus signs are added to show that in the compound the hydrogen is electropositive, and the radical is electronegative.

(2) Physical state of acids - acids may be gases as HCl or H_2S, or liquids as H_2SO_4, or solids as H_3BO_3.

(3) Chemical properties of acids:

(a) All acids have a sour taste when in solution.

(b) The hydrogen of acids is liberated by treatment with a metal:

$$HR + Me = MeR + H$$

(The symbol, Me, represents any metal. MeR means a salt of the acid with the same metal)

(c) Acids react with bases characteristically:

$$HR + MeOH = MeR + H_2O$$

(The formula MeOH represents the hydroxide of any metal).

(d) Acids have characteristic reactions with indicators. Acids change blue litmus paper to red. Acids change methyl orange from yellow to pink, etc.

b. Hydrogen ion - The H^+ of an acid is called a hydrogen ion; and it is the hydrogen ion that gives acids their chemical properties. We see from the symbol that hydrogen ion is an atom of hydrogen carrying a positive charge of electricity. We will learn later that it is the H^+ and only the H^+ which gives acids all of their properties, that the "strength" of acids is measured by the amount of hydrogen ion they are able to produce in solution, and that any compound that is able to liberate hydrogen ion is acidic whether or not its actual formula conforms to the above general one. For the present, however, we will hold to the general formula HR, as our definition of an acid.

15.

15. Bases - Another large group of chemicals is the one called "bases", and the properties which they posses in common are called basic properties. This group is the one commonly referred to as alkalies, or caustic alkalies, or simply hydroxides.

a. Common properties of bases:

(1) Structure - all bases are hydroxides of metals. They have the general formula MeOH, in which "Me" represents any metal. To show the nature of the compounds the general formula is written $Me^+ OH^-$, in which Me^+ represents any metallic ion and OH^- represents hydroxyl ion. The group - O - H, commonly called hydroxyl ion is very interesting. It is this ion that gives bases all of their properties, and as we are soon to learn it is the exact complement of hydrogen ion, and in many ways may be considered as its opposite. As in the case of hydrogen ion, we also find that any substance which is capable of releasing hydroxyl ion when it is dissolved in water, will have basic properties, whether or not the substance actually is a base.

(2) Chemical properties of bases:

All bases have bitter taste when dissolved. Bases react characteristically with acids. (MeOH + HR = MeR + H_2O. Metals are, in general, unaffected by treatment with bases, unless some other agent is present. Bases give characteristic reactions with indicators. Bases turn red litmus paper blue. Bases turn methyl orange yellow. Bases turn phenolphthalein red.

16. Neutralization - we have seen that acids have certain specific properties, while bases have a different set of properties, just as specific, and in some ways opposite. When substances having acidic properties are mixed with substances having basic properties in the proper proportion (molecule for molecule), both the acidic properties and the basic properties disappear, the solution is no longer sour or bitter, and if an indicator has been added to the solution it will have neither the color it has in acidic solutions nor that which it has in basic or alkaline solutions.

This reaction, neutralization, is a very common and a very important one. It is shown above under the paragraphs on acids and bases. It is repeated below, with the electrical charges shown, both in general and in a specific neutralization:

$$Me^+OH^- + H^+R^- = MeR + H_2O$$

$$Na^+OH^- + H^+I^- = NaI + H_2O$$

$$OH^- + H^+ = H_2O$$

It will be noticed that in every neutralization reaction water is formed, and moreover, as the third reaction emphasizes, that the water is formed by the combination of hydrogen ion and

hydroxyl ion. Some of each in a free state exist in water, but as they are present in equal amounts, their presence is now shown by the appearance of either basic or acidic properties. Similarly we call any other substance neutral which has every hydrogen ion counterbalanced by a hydroxyl ion. In fact, this balance of hydrogen ion and hydroxyl ion is neutrality.

In the second equation shown above: if molecular proportions of each chemical have been used, the solution will be neutral, all of the positive charges will have become balanced by negative charges. But in addition, a new substance is formed, namely, NaI, or sodium iodide. This is the other characteristic of neutralization reactions, - salt formation. We shall further discuss salts in the next paragraph.

To summarize, in a neutralization, four things always happen:

a. A substance having acidic properties reacts with one having basic properties.

b. Both acidic and basic properties disappear.

c. Water is formed.

d. A salt is formed.

17. Salts - salts comprise another large group of chemicals. Salts are composed of one or more atoms of a metal combined with one or more atoms of non-metals. They have the general formula MeR.

The properties of metals are too many and varied to attempt to describe, beyond saying that they are neutral in comparison to acids and bases, and relatively stable. This is to say, they do not react with as many other substances or react as readily as acids or bases. Most of the ordinary chemicals used in medicine are salts.

a. Normal salts are salts which are formed by the complete neutralization of acids and bases. They may be recognized at once from their formulas. If the formula contains neither hydrogen nor oxygen (apart from the oxygen of the electronegative radical) the salt is a normal one or a neutral one, as they are sometimes called. All of the following are normal salts. They contain none of the hydrogen from the acid and none of the hydroxyl from the base.

NaCl	Sodium Chloride	$Al_2(SO_4)_3$	Aluminum Sulfate
KBr	Potassium Bromide	$BiCl_3$	Bismuth Trichloride
LiI	Lithium Iodide	$SnCl_4$	Stannic Chloride
NH_4F	Ammonium Fluoride	$MgSO_4$	Nagbesuyn Chloride
CaCl2	Calcium Chloride	$AgNO_3$	Silver Nitrate
$BaSO_4$	Barium Sulfate	$Ca(NO_3)_2$	Calcium Nitrate
$Cu_3(PO_4)_2$	Cupric Phosphate	$U(NO_3)_6$	Uranium Nitrate

b. <u>Acid Salts</u> - some acids have more than one hydrogen ion per molecule; e.g., sulfuric acid H_2SO_4, and phosphoric acid H_3PO_4. It is possible to replace only one of these hydrogen ions with a metal; or in other words it is possible to only partially neutralize these acids. A salt formed by such a partial neutralization is termed an "acid salt." Any acid salt, shows in its formula, part of the hydrogen of the acid. Sodium Acid Sulfate is $NaHSO_4$; Potassium Acid Sulfide is KHS; Sodium Acid Carbonate is $NaHCO_3$; the following equations show the formation of these three acid salts, along with the formation of neutral salts of the same bases and acids:

$$2NaOH + H_2SO_4 = Na_2SO_4 + 2H_2O$$
$$NaOH + H_2SO_4 = NaHSO_4 + H_2O$$
$$2KOH + H_2S = K_2S + H_2O$$
$$KOH + H_2S = KHS + H_2O$$
$$2NaOH + H_2CO_3 = Na_2CO_3 + 2H_2O$$
$$NaOH + H_2CO_3 = NaHCO_3 + H_2O$$

Acid salts are either so named, or the syllable bi - inserted in the name to indicate that the salt is an acid one. In each of the following, the name in the two columns refers to the same salt:

Sodium Acid Sulfate	Sodium Bisulfate
Potassium Acid Sulfide	Potassium Bisulfide
Sodium Acid Carbonate	Sodium Bicarbonate
Ammonium Acid Silicate	Ammonium Bisilicate

(1) Acids which form acid salts: we have not so far referred to the basicity of acids. By basicity is meant the number of basic valence bonds the acid is capable of satisfying. This is exactly saying the same thing as that basicity is the number of H+ atoms the acid contains. We say that any acid which has one positive H atom is monobasic, or that it will satisfy one molecule of a base containing a monovalent basic element. Monobasic acids having only one positive hydrogen ion can form only normal salts, but as the number of hydrogen atoms increases, the number of possible acid salts increases. Thus, dibasic acids,

like sulfuric or carbonic acids have a series of normal and one series of acid salts. Tribasic acids, like phosphoric and boric acids, have a series of normal, and two series of acid salts. This is illustrated as follows:

$$3NaOH + H_3PO_4 = Na_3PO_4 + 3H_2O$$
$$2NaOH + H_3PO_4 = Na_2HPO_4 + 2H_2O$$
$$NaOH + H_3PO_4 = NaH_2PO_4 + H_2O$$

With three salts so similar, one immediately wonders how they are named to differentiate them. It is a problem that is solved only by completely stating what the salt contains. This is not always done, and when it is not, confusion remains. In some cases a logical definition clarifies the matter somewhat, as is the case with the three sodium phosphates. Several possibilities are shown below. Only those in the first column are self-evident:

		U.S.P. Names
Trisodium Phosphate	Tribasic Sodium Phosphate	
Disodium Hydrogen Phosphate	Dibasic Sodium Phosphate	Sodium Biphosphate
Sodium Dihydrogen Phosphate	Monobasic Sodium Phosphate	Sodium Phosphate

c. Basic Salts - basic salts while they are just the opposite of acid salts, are not so easily explained from a standpoint of neutralization. In general, a basic salt is one containing more base than is necessary for the formation of normal salts.

The base which remains in the salt is not so easily identified as the H+ of an acid salt, due chiefly to the tendency of two -OH radicals to decompose to a molecule of H_2O and an = O. We will, therefore, find either hydroxyl or oxygen from the base in the formula of a basic salt.

Note in the examples given below that basic salts may have their formulas written in two ways:

Basic salts of triatomatic bases:

Basic Ferric Chloride	Fe_2OCl_4
Basic Aluminum Chloride	Al_2OCl_4
Basic Bismuth Nitrate	$Bi(OH)(NO_3)_2$

Basic Ferric Chloride	$Fe_2O_2Cl_2$
Basic Aluminum Chloride	$Al_2O_2Cl_2$
Basic Bismuth Nitrate	$Bi(OH)_2NO_3 = BiONO_3 + H_2O$

Basic salts of diatomic bases:

Basic Lead nitrate	$Pb(OH)NO_3$	or $Pb(NO_3)_2.Pb(OH)_2$
Basic Mercuric Sulfate	$Hg_3O_2SO_4$	or $HgSO_4.(HgO_2)_2$
Basic Magnesium Carbonate	Mg_2OCO_3	or $MgCO_3.MgO$

(1) It may be seen that the naming of basic compounds is difficult, if one is to differentiate the compounds by name only. From the few examples above, whose names are given only in a general way, several ambiguities may be noted. We will not, however, go further into the nomenclature of these compounds at this time, for we will see later that the basic compounds which are used in medicine are mixtures of the several possible basic compounds in almost every case.

d. Double salts are salts formed when two different bases go to neutralize a dibasic acid. They are comparatively simple, and fairly common; e.g., Potassium and Sodium Sulfate, $KNaSO_4$, Sodium and Potassium Carbonate, $NaKCO_3$, Silver and Sodium Sulfate, $AgNaSO_4$.

18. <u>Chemical change</u> - Chemical changes are of fundamental interest to us. We outlined the scope of chemistry as the study of composition and of changes in the composition of substances. But the composition of substances cannot be studied without studying changes in composition; all analyses involve chemical change; all syntheses involve chemical change. In fact, we have not been able to discuss the little matter we have thus far undertaken without bringing chemical change into the picture to explain ourselves.

a. <u>Types of Chemical Change</u> - there are six different ways in which chemical reactions take place:

(1) <u>Direct Combination or Addition Reactions</u> - In addition reactions, two or more elements unite to form a compound, without the formation of any by-product. It may be illustrated by the following reactions:

$$Na + I = NaI$$
$$Mg + O = MgO$$
$$2Fe + O_3 = Fe_2O_3$$

(a) General formula for direct combination or addition reactions -

$$A + B = AB$$

(2) Simple Decomposition - Simple decomposition isn't always as simple as the name would seem to indicate. The term simple is used to differentiate this type of change from double decomposition, with which you now have more than a passing knowledge in the laboratory. What is meant is that one compound breaks down into two or more simpler compounds. It will be seen to be the exact opposite of addition reaction. The following examples illustrate various types of simple decomposition:

Any oxide of a "noble metal" breaks down when heated as follows: $Ag_2O = Ag_2 + O$. Carbonyl chloride, when heated, decomposes into a simpler compound and the element, chlorine: $COCl_2 = CO + Cl_2$. Calcium Carbonate, or chalk, when heated, breaks down into two simpler compounds: $CaCO_3 = CaO + CO_2$.

(a) General formulas for simple decomposition: the three reactions above are merely specific applications of the three general reactions below:

$$AB = A + B$$
$$ABC = AB + C$$
$$ABC = AB + BC$$

(3) Displacement - a third kind of reaction, already familiar from our study of acids, is the kind of reaction in which an element replaces some other element in a compound. Any metal which reacts with an acid, displaces hydrogen from the acid and takes the place of the hydrogen in the compound:

$$2\ Hg + 2\ HNO_3 = 2\ HgNO_3 + H_2$$

(a) General formula for displacement reactions: $AB + C = AC + B$.

(4) Double Decomposition (metathesis) - double decomposition is the type of reaction we have been carrying out in the laboratory, where the elementary atoms "change partners", e.g.,

$$HgCl_2 + 2KI = HgI_2 + 2KCl$$
$$2FeCl_3 + 3H_2S = Fe_2S_3 + 6HCl$$

(a) General Reaction for Double Decomposition: $AB + CD = AC + BD$.

(b) Neutralization which, we have already discussed, is a kind of double decomposition.

(5) Oxidation - a fifth type of reaction may possibly be better discussed at a later time. We will, however, mention here: Oxidation literally means adding oxygen to a compound; and in this sense, the reaction $Mg + O = MgO$, which we discussed as an addition reaction, is an example of an oxidation as well. The term is used also in a larger sense. Any reaction in which the oxygen content of a compound is increased, or in which the valence of an atom is raised, is an oxidation reaction. These will be discussed in greater detail at later dates.

(6) Reduction - reduction is the opposite of oxidation. It is a type of reaction in which oxygen is abstracted from compounds or in which the valence of an atom is lowered by chemical means. Reduction is carried out by reducing agents, many of which are important drugs and will be discussed as we meet them. It should be noted even at a first glance at reduction reactions that they frequently involve hydrogen in a similar manner to the way oxidation reactions involve oxygen.

b. Conduct of Chemical Reactions - in conducting chemical reactions there are many things to be considered. Perhaps chief among these is why we are carrying out the reaction, which should always be clearly in mind. More material considerations are (1) what substances take part in the reaction; (2) how much of them is to be used; and (3) under what physical conditions the reaction is to be carried out.

(1) Reagents - Reagents are the substances which enter the reaction and if we are to have a reaction, we must, of course, have reagents that will produce the change that we are aiming for in carrying out the reaction. Along the same lines, a further consideration is that we choose reagents which will produce the desired change in a way that we can use the result. To use a more specific example, if we are aiming to manufacture a chemical, we must choose reagents which will give us our compound in a way in which we can recover it. If we are analyzing a substance, we must choose a reagent which will cause a change which we can note, and, if possible, measure in some way. It is only by experience with similar reactions, or by experiment with a particular one, that we can tell what will be the best reagent in a specific case. Let us further illustrate by means of an example, and suppose we are striving to make lead nitrate. In casting about for various reactions that might produce the result, a chemist would certainly consider the following:

$$Pb + 2HNO_3 = Pb(NO_3)_2 + H_2$$
$$PbO + 2HNO_3 = Pb(NO_3)_2 + H_2O$$
$$PbCO_3 + 2HNO_3 = Pb(NO_3)_2 + H_2O + CO_2$$

All of these reactions result in the desired salt, lead nitrate. In the first, the by-product is a gas, which removes itself from the solution. In the second, the by-product is only water. In the third, water and a gas are produced. In any of these reactions, the by-product may be easily and completely removed. If we actually try, we will find that the first reaction is carried out only with difficulty, and consumes a great deal of heat, while either the second or third goes on spontaneously at room temperature. So we eliminate the first. To choose between the second and third, we need consider only which of the substances are at hand or are easily to be obtained. Price may also be a factor, in which case we find PbO the cheaper. It is also more readily available, particularly in the Army.

It is sometimes more difficult to choose reagents, but in any case, the reasoning or experiment must follow some such course as that above.

(2) Quantities of Reagents - how much of the reagents we are to use in a specific case may be calculated if we can write an equation for the reaction. This involves a use for chemical equations which we have not yet touched on and we can best discuss it by considering some specific reaction. Suppose our purpose is carrying out a reaction to produce 100 Gm. of mercurous iodide. To choose reagents with which we are familiar, let us prepare the iodide from mercurous nitrate and potassium iodide according to:

$$HgNO_3 + KI = HgI + KNO_3$$

Now the thing we have not considered about chemical equations is that they represent relative weights of the substances involved, being composed of the formulas of the compounds involved. These relative weights total the same on the two sides of the quality sign in just the same way that the various atoms must reach the same total. In the following the equation is expanded by showing the relative weights involved:

$HgNO_3$ +	KI =	HgI +	KNO_3
200.6	39.1	200.6	39.1
14.0	126.92	126.92	14.0
48.0			48.0
262.6	166.02 =	327.52 +	101.1

By simple arithmetic, it may be seen that the equation is mathematically true, each side totaling 428.62. (Does this satisfy the Law of the Conservation of Mass?)

Our problem now is to change the relative weights to actual weights which we can measure. This we can easily do by the use of proportion, for the actual weights involved are certainly proportional to the relative weights. Then:

$$HgNO_3 : HgI :: 262.6 : 327.52$$

and since we want 100 Gm. of HgI, we substitute that figure and solve:

$$HgNO_3 : 100 \text{ Gm.} :: 262.6 : 327.52$$

$$HgNO_3 = \frac{262.6 \times 100 \text{ Gm.}}{327.52} = 80.18 \text{ Gm.}$$

Similarly, for potassium iodide:

$$KI : HgI :: 166.02 : 327.52$$

$$KI : 100 \text{ Gm.} :: 166.02 : 327.52$$

$$KI = \frac{166.02 \times 100 \text{ Gm.}}{327.52} = 50.69 \text{ Gm.}$$

So we use 80.18 Gm. of mercurous nitrate and 50.69 Gm. of potassium iodide to make 100 Gm. of mercurous iodide, and we have as a by-product (80.18 + 50.69) - 100 Gm. or 30.87 Gm. of potassium nitrate in solution.

(3) Physical Conditions Surrounding Reactions - having decided what reagents are to be used, and in what quantity they are to be used, there yet remains to decide the conditions under which the reaction is to be carried out, that is to say, how the reagents are to be used. Here we have considerably more leeway than in the previous steps; but we cannot hope to throw the dry reagents into a beaker and take out dry mercurous iodide. In order for a reaction to occur, we must bring the reagents into intimate contact, for chemical reactions take place between atoms, not between lumps, or crystals, or bottlefulls. Since both the salts are soluble, we dissolve them ad mix the solutions. The mercurous iodied precipitates and may be washed until free of potassium nitrate and then dried.

Not every reaction goes along so simply, however, and there are often many other things to be considered. Even in the fairly simple reaction we have chosen as an illustration we can vary the nature of our precipitate somewhat by altering the conditions under which the reaction takes place. If hot, concentrated solutions of the salts are made and mixed, a heavy, dense precipitate will result; while if dilute, cold solutions are to be used, a lighter powder and a more finely divided one results.

Some of the factors which, in general, are used to control or modify the speed of reaction, or the degree of completeness to which a reaction will take place are shown below:

(a) <u>State of Aggregation</u>

Gases react most rapidly due to their molecules being in a state of rapid motion which results in an intimacy of contact throughout the mass of the gas.

Liquids react less actively than gases, but since they are readily mixed intimately, reactions between liquids take place fairly rapidly.

Solids, since they are rigid, react only at the surface, and often very slowly and incompletely. Again this is due to the fact that there is not intimate contact between the particles.

(b) <u>Temperature</u> - Temperature has a large effect both on the speed of the reaction and upon the degree of completeness to which the reaction takes place. Both the speed of reaction and the degree of completeness of reaction are, in general, increased by increasing the temperature. This also follows from the kinetic theory, the heat increasing the energy of the molecules.

(c) <u>Concentration</u> - by concentration is meant the actual number of molecules in a definite amount of space or volume. Increasing the concentration, naturally increases the molecular contact and speeds up reaction.

Concentration has another effect, which is used in some reactions which do not ordinarily proceed to 100% completion. In these cases, a complete reaction is often obtained if one of the reagents is present in excess, i.e., in greater concentration than the other.

(d) <u>Removal of End-Products</u> - Frequently the speed of reactions is increased if the products of the reaction are removed as they are formed. Some reactions may be carried to completion only by this means.

(e) <u>Catalysts</u> - Catalysts are substances, which by their presence cause reactions to proceed at a greater speed or to a greater extent than they would if the catalyst were not present. At first the action of catalysts was surrounded by much mystery and guess-work, but their use can nearly always be explained by sound physical or chemical reasoning. There are, nevertheless, no general rules one can learn regarding the action

of catalysts except in comparatively isolated reactions or types of reactions.

(f) <u>Pressure</u> - the effect of pressure is similar to that of heat, in increasing the sphere of contact of the molecules. It has little effect, however, on reactions between solids and liquids, in which gases are not involved.

(g) <u>Electricity</u> - Electricity has many and varied uses in connection with chemical reactions, too many for consideration here. It can be used to begin, to speed, or to complete reactions, and it acts quantitatively. A given amount of electricity will produce a given amount of chemical change. In fact, one of the measures of electricity, is the amount of chemical change it will produce.

c. <u>Reversible Reactions and Chemical Equilibrium</u> - from many of the statemets above, it will have been seen that not all chemical reactions proceed until all of the reagents are completely changed to new compounds, as the chemical equation shows. Reactions which do not go to completion are known as reversible reactions, and the point at which they seemingly stop is known as chemical equilibrium. These are more fully explained below, and as well in Simons, page 129.

All of the reactions carried out in the laboratory have gone to completion, as do nearly all of the reactions we have discussed in class. For example, the reaction, $HgCl_2 + 2KOH = HgO + H_2O + 2KCl$, goes on until all of the mercury bichloride is used up, or all of the potassium hydroxide, or both, depending on the concentration of the two reagents. When either or both of the reagents is used up, there can be more HgO formed because there is no more material left to make it, and the reaction stops. To use a different example, one with which we are all familiar: $C + C_2 = CO_2$, representing the burning of coke (carbon) in a stove or furnace, the coke continues to burn (react) until it is all used up, or until the air (oxygen) supply is cut off. If either happens, the fire goes out (the reaction stops).

In reversible reactions, however, not all of the reagents are permanently changed to new products; but after a certain proportion of the reagents has reacted, an apparently stable mixture results. As an example of a reversible reaction let us consider: $2\ NaCl + H_2SO_4 \rightleftharpoons 2\ HCl + Na_2SO_4$. If sulfuric acid is added to salt, some of the salt is converted to hydrochloric acid, but not all of it is. In other words, if we used molecular quantities of the reagents, we should expect to find only the two products, sodium sulfate and hydrochloric acid, in the solution. Instead we find four: sodium chloride, sodium sulfate, hydrochloric acid, and sulfuric acid. Moreover, if repeated analyses of this

solution are made at various times but at the same temperature, they show that the four substances are present each in the same concentration, at each of the analyses. We say that a state of chemical equilibrium (balance) has been reached.

We know, as a result of the kinetic theory, that the reaction is not just at a standstill, as it seems to be, but that it has reached a point at which just as many atoms are changing one way as there are changing back again. In other words, the reaction is proceeding in both directions at the same time. That is the reason the reaction is called reversible, and also the reason that the equality sign is modified to become an arrow pointing in each direction. It is also the reason for the state of balance or equilibrium. It is equally true that if we start with the substances on the right of the equality sign, instead of those on the left, we get the same equilibrium mixture, if the temperature is the same.

There are several ways to complete these reactions, as has been intimated in discussing conditions under which substances react. If the concentration of one of the reagents is considerably more than the other, the reaction proceeds to a greater extent; and, if one of the end-products is taken out of the solution, the same thing happens.

19. Physical Properties and their Importance in Chemistry - Properties have already been described to some extent as the means by which substances make themselves evident to our senses. They have already been divided into general and specific properties, according to whether or not they are possessed by all substances to the same degree. Another useful division of properties is according to whether or not they are measured by physical or chemical means. As chemical properties are our main interest in this course, we shall not attempt to discuss them in general here, beyond stating that frequently we are unable to measure a chemical property except by physical means. For this reason we are compelled to first learn something about physical properties.

a. Physical Properties Possessed by all Substances - In this paragraph, an attempt is made to define and describe in general those properties, often called "PHYSICAL CONSTANTS" which are possessed by practically all substances, regardless of their state of aggregation or chemical nature. No absolute separation of this sort can be made since many of these properties are carried over from one state to the other; but a little later the properties possessed only by gases, those by liquids, and those by solids, will be treated separately.

(1) Specific Gravity - Specific gravity is merely a comparison of the weight of two substances. Let us not surround it with any mystery, and attempt to make it difficult. All of us are familiar with the statement that lead is heavier than feathers. This is a statement of specific gravity, and other statements of specific gravity are just as simple, if we really understand this one. All the scientific definition of specific gravity does is to make the statements uniform for large groups of substances. It does this by reducing the statement of specific gravity to a mathematical ratio, a fraction. This ratio or fraction is:

$$\frac{\text{Weight of some volume of substance "A"}}{\text{Weight of an equal volume of substance "B"}}$$

Let us examine this for lead and feathers, in order to go back to our illustration. Suppose we take a bucket and fill it with lead and weigh it, finding the weight, say 256 pounds. If we repeat the same procedure with feathers, we find the weight is 7 pounds. But this weight includes the bucket. If we weigh the bucket empty and find it weighs 6 pounds, we can subtract this amount from the other two weights and find how much the lead alone and the feathers alone weighed. The specific gravity then is the ratio:

$$\frac{250}{1}$$

Or we say that lead is 250 times as heavy as feathers.

(2) Specific gravity compared to water - the use of water as a basis of comparison for specific gravity has become so common that when the term specific gravity is used without stating the basis of comparison, it is understood that comparison to water is intended. There are many reasons for this: the figures obtained are in the neighborhood of 1; water is easily obtainable; water does not change as much with temperature as some other substances; figures obtained with it are interchangeable, in the metric system, with density, and many others.

For ordinary or common specific gravities, then our fraction always has as a denominator the weight of an equal volume of water, or:

$$\text{Specific Gravity} = \frac{\text{Weight of some volume of substance "A"}}{\text{Weight of an equal volume of water}}$$

Water is used as a basis of comparison for all liquids and solids and may be used for gases as well.

(2) Density - Density is frequently confused with specific gravity. It is the relation between the weight of a substance and the volume it occupies. To state the same thing mathematically:

$$\text{Density} = \frac{\text{weight}}{\text{volume}}$$

The reason for the confusion between density and specific gravity is the peculiar set-up of the metric system of weight and measure, by which volumes are measured by the amount of space occupied by 1 Gm. of water. In the metric system then, the denominator of the fraction will be the same whether we are computing density or specific gravity. Let us illustrate this by determining both the specific gravity and the density of alcohol.

Let us suppose we weigh the contents of a glass of water, then the contents of the same glass of alcohol, then measure the contents by pouring them into a graduate:

Weight of glassful of water	200.0 Gm.
Weight of glassful of alcohol	163.2 Gm.
Volume of glass	200.0 cc.

$$\text{Specific gravity of alcohol} = \frac{163.2}{200.0} = 0.816$$

$$\text{Density of alcohol} = \frac{163.2 \text{ Gm.}}{200.0 \text{ cc.}} = 0.816 \text{ Gm/cc}$$

In specific gravity the relation is between weights alone and it makes no difference what system we use, the specific gravity will be the same. In density we are dividing weight by volume, and we cannot logically drop the units of measure. They remain in the result. Densities calculated in some other system will be different from those calculated in the metric system. An engineer will tell us that the density of water is 64.4 pounds/cubic foot; some pharmacists say that it is 454.6 grains/fluid ounce; while we ordinarily say it is 1 Gm./cc. The only difference is in the system of weight and measure used. It is, however, important to express the units of measure whenever confusion might result.

(3) Solubility - Solubility is the extent to which a substance dissolves in a solvent. Like specific gravity, we speak of the solubility of a substance as the extent to which it is dissolved by water, unless we specifically designate some other solvent. There are two methods of stating solubilities: (1) as the number of cc. of solvent required to dissolve a fixed amount of a substance, or (2) as the amount of a substance which can be dissolved by a fixed amount of a solvent. The U.S.P. uses the first method, and we shall prefer its use, although there is little difference.

b. Specific physical properties of gases - There are several properties almost always discussed when any gas is being considered. Although these are of little direct application to laboratory work, we may find at some time that we wish we knew at least what these properties mean. Then, in addition, there are more and more gases being used in medicine.

(1) Specific gravity - Specific gravity of gases is the same as other specific gravities, except the specific gravity of a gas may be compared to another gas. The gases used for comparison are air and hydrogen. When these other gases are used as standards for the specific gravity, a statement to that effect usually accompanies the figure. These statements are usually in the form "A = 1" meaning compared to air, or "H = 1" meaning compared to hydrogen.

(2) Density - the density of gases is frequently given as Gm. per liter, because the figures obtained when Gm./cc are used run to several decimal places. Again, there is no confusion if the statement is complete.

(3) Solubility-due to the nature of gases, one sees solubility of gases expressed in parts by volume.

(4) Critical Data - Critical date of gases refer to the ease with which gases may be liquefied. The critical temperature of a gas is the temperature above which a gas cannot be liquefied no matter how much pressure is applied to it. And the critical pressure is the pressure at which the liquefaction begins when the gas is maintained at its critical temperature. Generally speaking, the higher the critical temperature and the lower the critical pressure of a gas is, the easire it is to liquefy.

c. Specific properties of liquids:

(1) Specific gravity

(2) Density

(3) Solubility - solubility of liquids is often given by volume.

(4) Boiling Point - the boiling point of a liquid is the temperature at which the liquid and vapor phases of a liquid remain in equilibrium at 1 atmosphere of pressure.

(5) Freezing Point - the freezing point or congealing point of a liquid is the temperature at which the liquid and its solid form remain in a state of equilibrium. Needless to say, this is the same as the melting point of a solid.

(6) Viscosity - this is the property of liquids by virtue of which they stick to surfaces. It is measured by the length of time it takes the liquid to drain from a fine tube.

(7) Surface Tension - surface tension is the property of forming a film of closely packed molecules at the surface of liquids.

d. Specific properties of solids:

(1) Specific Gravity

(2) Density

(3) Solubility

(4) Crystalline Form - the crystalline form of a solid is a description of the shape taken by its crystals when it is allowed to crystallize. These forms are very definite and always the same for the same solid under the same conditions. The study of them is a science in itself, and for our purposes the mere statement that the substance is crystalline or amorphous (not crystalline) is usually sufficient.

(5) Melting Point - see freezing point above.

20. Ionization - Dissociation of compounds upon solution, or as the phenomenon is generally called, ionization, is of the utmost importance to all concerned with medicine and laboratory and as only a brief treatment of the subject is given here, the student is expected to master all of it and to gradually become able to apply all of the matter to extemporaneous problems of compounding as they come up. This is no small task, and in the limited time available will require the best efforts of all concerned.

a. General - Dissociation, it will be remembered, was one of the general types of chemical reaction; the following general reactions were given as examples: (simple decomposition)

$$AB = A + B$$
$$ABC = AB + C$$
$$ABD = AB + BC$$

It has been found that many substances on being dissolved in liquids, especially on being dissolved in water, undergo dissociation and remain dissociated as long as they are in solution. Such substances, which dissociate on being dissolved are called electrolytes because they are the same substances which increase the capacity of water to conduct electricity.

It was long ago discovered that these same substances deviated from non-electrolytes in the degree to which they changed the properties of solvents used to dissolve them. Electrolytes produce a greater depression of the freezing point, a greater elevation of the boiling point, and a higher osmotic pressure in solutions of them. Since it is known that these properties are proportional to the number of particles dissolved in a given amount of liquid, there was nothing left to believe except that electrolytes split into smaller particles and a greater number of them. This gave rise to the theory of electrolytic dissociation, which states that MOLECULES OF ELECTROLYTES WHEN DISSOLVED IN WATER, BREAK UP TO A VARYING DEGREE INTO INDEPENDENT PARTICLES CHARGED WITH ELECTRICITY AND THE NATURE AND NUMBER OF THESE PARTICLES DETERMINE, TO A LARGE DEGREE, CERTAIN PHYSICAL AND CHEMICAL PROPERTIES OF THE SOLUTIONS. These independent charged particles are called IONS.

b. Properties of ions as compared to atoms -

(1) Kinds of Ions - Ions are of two kinds, different as regards the nature of the electrical charge on them:

CATIONS - or electro-positive ions have a positive charge on them, and when an electrical current is applied to a solution, the cations move toward the cathode or negative pole.

ANIONS - or electro-negative ions have a charge of negative electricity on them and upon application of a current, move toward the anode or positive pole.

(2) Relation between anions and cations - neutrality of solutions - in every solution, anions and cations are present in equal numbers - for each anion, one cation; for each positive charge, one negative charge. It is for this reason, that the solution as a whole is electrically neutral. Moreover, while the statement of the electrolytic theory describes ions as "independent particles," they are never present without the corresponding oppositely charged ion, so that they should not really be said to be independent particles.

(3) Ions are atoms plus a charge of electricity - Ions react according to electrical laws rather than to chemical laws: they unite with other ions only if the other ions are oppositely charged. Thus, while two chlorine atoms will react to form a molecule, two chlorine ions will not, unless they are first removed from solution. In other words, before ions can react like atoms, they must become atoms. Moreover, in the case of many ions, there would be a reaction between the ion and water, if the ion were an atom. For instance, take the dissociation of sodium chloride in solution. If the sodium ion were the same as the sodium atom, it would react with the water to form a molecule of NaOH and hydrogen would be given off. Sodium chloride solutions do not do this unless sufficient electrical force is applied to remove the ion from solution. In the same solution, if the chlorine were in the form of atoms, it would color the solution yellow, which it does not.

c. Symbols for ions - the symbol for an ion is the symbol for the corresponding atom with the proper electrical charge added, as a plus or minus sign. Thus the symbol for the chlorine ion is Cl^-, for the sulfate ion it is SO_4^{--}, for the sodium ion Na^+, for the ferrous ion Fe^{++}, and for the ferric ion Fe^{+++}. From these symbols it may be seen that the charge on an ion is proportional to its valence. This has already been applied practically in the laboratory in balancing chemical equations.

d. Dissociation reactions - it was stated at the beginning of this discussion that dissociation in solution was a chemical reaction. It is, moreover, a reversible reaction, which means that it does not, in all cases, go to completion, but reaches a state in which there are as many molecules dissociating into ions as there are ions reuniting to form molecules , chemical equilibrium, in other words. As with all reversible reactions there are physical conditions which influence the composition of the "equilibrium mixture." The condition which has the most influence is concentration, i.e., the relation between the number of solute and of solvent molecules. Ordinarily, dilute solutions ionize to a greater extent than more concentrated ones of the same solute and solvent.

(1) Equations - Ionic equations are written as other reversible reactions, using the symbols for ions. Usually each ion is shown separately:

$$HCl \rightleftharpoons H^+ + Cl^-$$

$$H_2SO_4 \rightleftharpoons H^+ + H^+ + SO_4^{--}$$

$$Ca(OH)_2 \rightleftharpoons Ca^{++} + OH^- + OH^{--}$$

It should be noted that radicals do not ionize within themselves to liberate the ions of each of the elements of which the radical is composed, but that the charge is on the radical as a whole, thus OH^- and SO_4^{--}.

To interpret the above a little more fully: the first reaction tells one that hydrochloric acid dissociates into hydrogen ion and chloride ion until a certain point is reached depending upon the concentration. At this point of equilibrium there are as many hydrogen and chloride ions reacting to form hydrochloric acid molecules as there are hydrochloric acid molecules dissociating into hydrogen ion and chloride ion. At this point there are three kinds of solute present: (1) hydrogen ion; (2) chloride ion; and (3) hydrochloric acid molecules.

e. Ionization constants - the ionization constant is a measure of the degree of completion of a dissociation reaction. It varies with concentration and with temperature; and it is too cumbersome a figure for use in ordinary laboratory work. In fact, ionization data are of little use to the practical pharmacist, since very little is yet known about the ionization of concentrated solutions. The student is referred to the electromotive series, and from the standing of an ion in the general series and experience with similar salts, he can usually estimate the degree of ionization closely enough; see paragraph on hydrolysis below.

f. Chemical reactions in the light of electrolytic theory - chemical actions in aqueous solutions are practically always reactions between ions. Some workers claim that reactions are always between ions. There remain, however, reactions between substances that ionize only slightly, if at all, so that theory cannot be altogether correct.

g. "Strength" of acids and bases - an attempt has been made throughout this course to use the term "concentration" to describe the weight of solute per unit of weight or volume of solvent in contrast to the term "strength." The term strength is used to refer to the intensity of acid or basic properties, which is a direct consequence of ionization. Acids which ionize to a large extent are called STRONG ACIDS; those which ionize

only slightly are called WEAK ACIDS, etc. To put the same statement a little differently, the acids which ionize most have the greatest concentration of hydrogen ion per unit of concentration, and acid properties are the result of hydrogen ion. Those bases which ionize to the greatest degree have the greatest concentration of hydroxyl ion and are therefore called the STRONG BASES.

21. Hydrolysis - hydrolysis means literally: splitting by water, and it has been defined as double decomposition between another substance and water. Like most phenomena, hydrolysis was known long before it was explained. Chemists early found that many substances, which of themselves are neutral, impart acid or alkaline reactions to water when they are dissolved. From early observations it was noticed that certain acids nearly always produced salts that gave solutions an acid reaction, and they called these acids strong acids. Similarly, it was noted that bases of certain metals produced salts with a tendency to impart basic reactions to solutions. They called these strong bases. Ionization gave these studies a new meaning, and it is in terms of ions that we will discuss hydrolysis.

It has already been pointed out that strong acids and bases are those which ionize largely, and that weak acids and bases are those which ionize only slightly. We note further that metals high in the electromotive series have strong bases, while metals lower in the series produce weak bases. As regards acids, most of the inorganic acids are relatively strong, most of the organic acids are weak acids. For the most part, the binary acids are stronger than ternary acids of the same element (sulfur is a notable exception).

22. Hydrogen ion concentration of water: in our discussion of chemistry we have seen how inorganic compounds break up or ionize in water. We are now going to take up a special ionization that has to do with the production of hydrogen ions.

Before doing this it will be well to review three things that we will use in our discussion. These are the definition of an acid, base and neutralization. The first is the definition of an acid which we might define by saying that it is any substance capable of liberating hydrogen ions, i.e., $HCl + H_2O = H^+ + Cl^-$, similarly a base might be defined as any substance capable of liberating hydroxyl ions, i.e., $NaOH + H_2O = Na^+ + OH^-$. Neutralization is the process in which an acid reacts with a base to form a salt and H_2O. For our purposes a consideration of the formation of water is the main point for we may consider our acid as H^+ and Cl^-, and the base as Na^+ and OH^-. When these react we have water (H_2O) and NaCl which in solution is Na^+ and Cl^-. Hence we may write our reaction as $H^+ + Cl^- + Na^+ + OH^- = H_2O + Na^+ + Cl^-$ or more simply
$H^+ + OH^- \quad H_2O$.

Keeping this in mind we can continue on our discussion with a treatment of the difference between total acidity and hydrogen ion concentration.

For an example, let us take some N/10 acid. Here we say that the total acidity is tenth normal for it will take exactly an equivalent amount of N/10 base to neutralize it.

Now let us consider the hydrogen ion concentration. In any ionization, the process is not totally complete. This is usually written as HCl $\qquad$ $Cl^- + H^+$ and called a "reversible process." This phenomenon is especially marked in weak acids such as acetic acid. In this case ionization occurs only to the extent of about 1%. This would mean that for every 100 molecules of acetic acid dissolved in water, only one would ionize to form hydrogen ions. Thus we could say that in N/10 acetic acid the total acidity was tenth normal and the hydrogen ion concentration was .1 x .01 or .001N. or N/1000.

We will now boldly define pH and then define the words used in the definition.

pH is the log of the reciprocal of the hydrogen ion concentration. From our discussion we already know what hydrogen ion concentration is, but to repeat it is merely the concentration of actual hydrogen ions in solution.

In order to define the term reciprocal we will merely have to recall a little of our grammar school arithmetic. To take the reciprocal of a number we invert that number or turn it upside down. For a fraction this is easy, for here we merely interchange denominator and numerator, i.e., 3/4; the reciprocal of it would be 4/3. To go on a little further, we will consider decimals. These of course are fractions in which the denominator is some power of ten, i.e., .1 can be written as 1/10 or .02 can be written as 2/100. Therefore, the reciprocal of .02 or 2/100 would be 100/2 or 50. To complete this discussion of inversion we will have to talk of whole numbers. We commonly write down a whole number, i.e., 50. If we now divide this whole number by one, it will not be changed. Therefore, we could say 50 = 50/1. If we do this, it is easy to take the reciprocal of it by taking 50/1, and turning it upside down, giving us 1/50.

We now have only one more term left to define. The definition of a log is just as simple as those we have just discussed, but not quite so familiar to you. Before we make our definition, though, let's have a little more arithmetic.

We all know that $100 = 10 \times 10$ or 10^2. Similarly, $1000 = 10 \times 10 \times 10$ or 10^3. Another way of saying this is that ten to the power two equals one hundred and ten to the power three equals one thousand. Now let us consider $10^{2.303}$. We know this will be greater than 10^2, or 100 and less than 10^3 or 1000. By the use of suitable tables we can find that $10^{2.303} = 200$. You are no doubt now beginning to suspect that any number can be written as ten to a certain power, and that is correct. The definition of a log follows directly from this fact, for a log is the power to which ten is raised to give that number.

We have now considered the meaning of all the words in the definition of pH, but no doubt it still doesn't make a great deal of sense, so let us see how it works. To do so, however, we will have to digress somewhat again.

It is well known that water instead of being merely H_2O is really a reversible reaction, i.e., $H_2O \rightleftharpoons H^+ + OH^-$. Further, it is known by experiment in this case that $(H+) \times (OH^-) = 1/10^{14}$. (The brackets () around the ion means "concentration of.") From the equation above it can be readily seen that for each molecule of water that ionizes, one ion of hydrogen is formed. Therefore, the concentrations are equal. Thus we can say $(H^+) \times (OH^-) = (H+)^2$; $(H+)^2 = 1/10^{14}$ or $(H+) = 1/10^7$. By recalling a little more arithmetic we write $1/10^7$. Reducing all the above paragraph into a nutshell, we can say that in neutral water $(H+) = 10^{-7}$.

Now let us recall our definition of pH. pH = the log of the reciprocal of the (H+), (hydrogen ion concentration). Let's see, in water $(H+) = 10^{-7} = 1/10^7$, the reciprocal of $1/10^7 = 10^7/1 = 10^7$, and the log of $10^7 = 7$.

23. ph

ph	
1	ACID SOLUTIONS
2	
3	
4	
5	
6	
7	NEUTRAL SOLUTIONS
8	ALKALINE SOLUTIONS
9	
10	
11	
12	
13	
14	

24. Titration -

a. It is merely a convenient method of measuring the amount of one solution that will react with a fixed amount of another solution. The fixed amount of the one solution is measured with a pipette into a beaker, and if necessary diluted with water. The standard solution, or titrating reagent is placed in a burette, and added carefully drop (gtt) by drop, until the reaction is complete. This is found by previously adding an indicator to the solution in the beaker which changes color when the reaction is complete, or commonly alluded to as when the "end point" has been reached.

b. The following table shows some of the most commonly used indicators, their color changes and the pH at which this change takes place:

Indicator	Color Change (Acid to Base)	pH Range
Methyl Orange	Pink to yellow	4.4 - 4.6
Methyl Red	Pink to yellow	4.8 - 5.4
Phenol Red	Yellow to red	7.0 - 7.4
Phenolphthalein	Colorless to red	7.8 - 8.0
Congo Red	Blue to pink	3.3 - 4.6
Litmus	Red to blue	5.0 - 6.0
Resorcin Yellow	Yellow to Orange	11.1 -12.7

25. Buffer Action - this is a term developed along with knowledge of hydrogen ion control. Briefly, buffers are substances which, when they are added to solutions, decrease the rapidity with which pH changes on dilution or on addition of hydrogen or hydroxyl ion. Buffer action is a sort of masking, by means of whick the total acidity or alkalinity of a solution may be altered without marked change of pH.

The mechanism of buffer action is difficult to explain without going into mass action phenomena; and it will suffice here to note that buffers act by selectively repressing or decreasing the extent of ionization. Remington (page 543) gives a more complete explanation of buffer action. Buffers are seldom of direct application in laboratory, except in the preparation of some solutions. Each of these is a problem in itself.

26. Types of Solutions:

a. Simple solution of reagents - is one in which a designated weight or volume of reagent is placed into a specified solvent. When no solvent is designated, distilled water

is always intended. Here caution is definitely to be observed in that the proper reagent is selected, also accurate weighing and measuring of both the reagents of the solute and solvent is to be observed.

b. Percentage solutions - in this type of solution simple arithmetic in percentage is the vital knowledge necessary in that all volumes are calculated on the basis of 100. And if this can be kept in mind, little cause for confusion will arise. Percentage solutions are rough calculations, hence Gms. and cc's are interchangeable. For example, a 1% can be made of any reagent by placing 1 Gm. or 1 cc and adding sufficient water to make 100 cc. Or in other words, 1 Gm. or 1 cc qs. to 100 cc. In fractions of % for instance, .25% can be made by weighing er measuring, 1/4 Gm., 250 mg., .25 cc qs. to 100 cc with water. In like manner, where the % is greater than one, such as a 20%. 20 Gms. or 20 cc is qsed up to 100 cc, as one would state in rather a common laboratory vernacular.

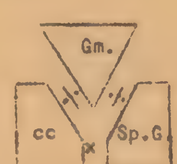
c. Normal Solutions - this type of solution is one containing one equivalent weight (chemical equivalent) of a reagent qs (i.e., as much as necessary of distilled water) to 1000 cc. (1 liter).

(1) An equivalent weight or chemical equivalent is the amount of a reagent that either contains or reacts with 1 atomic weight of hydrogen, 1.008 Gm., this to be expressed in Gms.

(2) The cautions to be observed in the making of this solution is the accuracy of calculation, namely, the accuracy of atomic weights, the addition of proper molecular weights, the accurate number equivalents, the conversion of Gms. to cc's, if the reagents are liquid, and the proper calculation of various assays of the reagents. As an example:

4N-H_2SO_4 - Sulfuric acid
H_2 = 2.016
S = 32.060
4O = <u>64.000</u>
98.076 ÷ 2, since there are 2 H's = 49.038 Gms. = 1 normal qs. to 1000 cc
4N = 49.038 x 4 = 196.152 Gms. qs to 1000 cc

cc x Sp G(specific gravity) = Gm.



Using the above equation, one can convert the calculated Gms. in 4N to the number of cc to be used, provided the Sp.G. is known. In the case of the particular H_2SO_4, the Sp.G. is 1.84. The solution then follows: 196.152 Gm. ÷ 184 = 106.604 cc qs to 1000cc.This would be correct if the assay of the H_2SO_4 would be 100%. In this case it is 96% to 98%. Hence 97% should utilize in its calculation thus: 106.604 ÷.97 = 109.901 cc qs to 1000 cc, will give an accurate 4N H_2SO_4.

d. Molar Solutions - is a solution containing the molecular weight in Gms. of the reagent qs to 1000 cc. with distilled water. Here no equivalent weight or chemical equivalent are necessary. However, conversion from Gms. to cc's and a calculation for percentage of assay should be effected.

e. Miscellaneous Solutions:

(1) Alcoholic Solution - is one in which the solvent is alcohol, ethyl, 95%.

(2) Aqueous Solution - is one in which the solvent is distilled water. And whenever no reference is made to the type of solvent, distilled water is intended.

(3) Isotonic Solution - is one having the same osmotic pressure as some other solution with which it is compared. Isotonic Salt Solution (.9%) has the same osmotic pressure as the salt solution in body cells or body tissue.

(4) Hypotonic Solution - is one having an osmotic pressure less than the comparing solution.

(5) Hypertonic Solution - is one having an osmotic pressure greater than the comparing solution.

BLOOD CHEMISTRY

I. Protein Free Filtrate

A. Solutions:

1. Sodium Tungstate, 10% ($Na_2WO_4 \cdot H_2O$)
2. Sulfuric Acid, .66N (H_2SO_4)
3. Sulfuric Acid, 10%
4. Potassium Oxalate, 2%
5. Benzoic Acid, .25%

B. Procedure:

1. 1 vol. of oxalated blood
   and
2. 7 vol. of distilled water (to lake blood), shake well
   add
3. 1 vol. of 10% $Na_2WO_4 \cdot H_2O$, mix
   add
4. 1 vol. of .66N H_2SO_4, shake
   (changes pink to dark brown), and if not add
5. drop by drop 10% H_2SO_4 until right color, filter.

C. Action:

The 2/3 N acid is intended to be equivalent to the sodium content of the tungstate so that when equal volumes are mixed substantially the whole of the tungstic acid is set free without the presence of an excess of H_2SO_4. The tungstic acid set free is nearly quantitatively taken up by the proteins and hence, the blood filtrates obtained are free of protein and only slightly acid.

II. Blood Sugar Determination

A. Solutions:

1. Stock Sugar Solution. 1% Anhydrous Dextrose Solution in .25% Benzoic Acid.

2. Working Standards:
   a. Weaker: 5 cc Stock Sugar Sol. + .25% Benzoic Acid qs. to 500 cc.
   b. Stronger: 5 cc Stock Sugar Sol. + .25% Benzoic Acid qs. to 250 cc.

3. Alkaline Copper Solution:

40 gm. Anhydrous Sodium Carbonate
400 cc. distilled H_2O in 1 liter flask
7.5 gm. Tartaric Acid, when dissolved add
4.5 gm. Crystalized Copper Sulfate
qs. to 1000 cc with Distilled H_2O.

4. Molybdate - Phosphate Solution:

35 gm. Molybdic Acid
5 gm. Sodium Tungstate
200 cc 10% Sodium Hydroxide Solution in 1000 beaker
200 cc distilled H_2O
Boil vigorously 40 minutes, then cool
Add distilled H_2O qs to 350 cc.
125 cc Phosphoric Acid (85% solution)
Add distilled H_2O qs to 500 cc.

B. Procedure:

1. 2 cc Protein Free Filtrate in blood sugar tube
2. 2 different standards in 2 blood sugar tubes
3. To all add 2 cc of Alkaline Copper solution
4. Boil for 6 minutes in boiling water bath
5. Cool for 3 minutes without shaking
6. 2 cc of Molybdate-Phosphate Solution
7. Add distilled H_2O up to 25 cc., mark
8. Invert several times, mix well.
9. Compare in colorimeter.

C. Calculation:

Weaker Solution:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \ (20) \times 100 = \text{mg. per 100 cc}$$

Stronger Solution:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \ (20) \times 200 = \text{mg. per 100 cc}$$

III. Blood Sugar Determination by Lewis and Benedict

A. Solution:

1. Picric acid, chemically pure. For each determination 0.5 Gm. will be required.

2. Sodium carbonate, saturated solution (22%). Each determination requires 2 or 3 cc.

3. Standard dextrose solution, a 0.02% solution of pure dextrose in saturated aqueous solution of picric acid.

B. Procedure:

1. Place 2 cc of oxalated blood in a centrifuge tube, preferably with round bottom to facilitate stirring; and add 8 cc of distilled water.

2. Mix well, and let stand until the blood is completely laked.

3. Add 0.5 Gm. of dry picric acid and stir well with a slender glass rod. Let stand for five minutes, with occasional stirring.

4. Centrifugalize, and filter the supernatant fluid through a small filter paper into a dry test tube.

5. Transfer 3 cc of the filtrate to a 10 cc volumetric flask (a tall test tube with a 10 cc mark or an accurately graduated centrifuge tube will answer). Add 1 cc saturated solution of sodium carbonate.

6. Place 3 cc of the standard dextrose-picric-acid solution in a similar flask or tube, and add 1 cc of saturated sodium carbonate solution.

7. Heat both tubes in a beaker of boiling water for fifteen to twenty minutes. Cool to room temperature.

8. Make up the unknown and the standard solution to 10 cc with distilled water. Mix well.

9. Compare the unknown with the standard in a colorimeter. The calculation is based upon the fact that the unknown represents 0.6 cc of blood and the color standard 0.6 mg. of dextrose. With the plunger type of colorimeter or the Denison Laboratory Colorimeter, the following formula may be used:

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 100 = \text{mg. of dextrose in 100 cc of blood.}$$

When the blood sugar is high, a standard containing double the amount of dextrose-picric-acid solution must be used, and this requires that the final result be multiplied by 2.

IV. Marshall Test: Determination of sulfanilamide, sulfapyridine, sulfathiazole, sulfaguanidine, and sulfadiazine in blood.

A. Solutions:

1. A solution of trichloracetic acid containing 15 gm. dissolved in water and diluted to 100 cc.

2. A 0.1% solution of sodium nitrite. Should be prepared fresh each day.

3.a. An aqueous solution of N-(1-naphthyl) ethylene-diamine dihydrochloride containing 100 mg. per 100 cc. This solution should be kept in a dark colored bottle. If kept in ice box when not in use, it will keep for one week. (See footnote)

4. A solution of saponin containing 0.5 gm. per liter.

5. 4N hydrochloric acid.

6. A solution of ammonium sulfamate, containing 0.5 gm. per 100 cc.

7. A stock solution of sulfanilamide, sulfapyridine, sulfathiazole, sulfaguanidine, or sulfadiazine in water containing 200 mg. per liter. The chemically pure, dry, finely powdered drug should be used, not tablets.

8. Working Standards - these are made according to the following table:

.5 cc stock, add 18 cc Trichloracetic Acid, qs to 100 cc = .01 = 2 equation factor.
1. cc stock, add 18 cc Trichloracetic Acid, qs to 100 cc = .02 = 4 equation factor.
2.5 cc stock, add 18 cc Trichloracetic Acid, qs to 100 cc = .20 = 10 equation factor.
5.0 cc stock, add 18 cc Trichloracetic Acid, qs to 100 cc = .10 = 20 equation factor.

(Footnote)
3.b. The alternate solution, and the one most frequently used is 1 cc of Dimethy-a-naphthylamine qs to 250 cc with Ethyl Alcohol 95%. Caution to be observed in the use of this solution is, that in the procedure 5 cc of this solution is used instead of 1 cc of solution 3.a. above.

B. Procedure:

1. 1 cc or 2 cc oxalated blood.

2. 15 cc or 30 cc Saponin Solution, .05%. Wait 2 minutes or until laked.

3. 4 cc or 8cc Trichloracetic Acid,15%; filter.

4. 10 cc Filtrate; also 10 cc of Standard

5. 1 cc Sodium Nitrite Solution, .1% same here. (This to be prepared fresh each day); wait 3 minutes.

6. 1 cc Ammonium Sulfamate Solution .5% same here; wait 2 minutes.

7. 5 cc Dimethyl-a-naphthylamin Solution same here. (1 cc of N-(1-Naphthyl) ethylenediamine dihydrochloride is used at times if available as an alternative solution).

8. The unknown is then compared with the appropriate standard, as treated above, in the colorimeter.

C. Calculation:

1. $\frac{\text{Reading of Standard}}{\text{Reading of Unknown}}$ x Equation factor (see 8,page 47) = mg.% of free sulfonamide

2. Total sulfonamide determination is obtained by using 5 cc of the filtrate in a calibrated test tube, and adding 1. cc of 4N-hydrochloric acid solution. The mixture is heated on a boiling water bath for 2 hours. Taking care not to allow the volume go below 3 cc, cool and adjust the volume to 10 cc. This gives the final dilution of blood 1:40. The subsequent diazotization procedure is as described above.

3. The use of conversion factors can be considered in the calculation, if standards are not of the same sulfonamide as the sulfonamide in the blood specimen. Some of the most frequently used factors are in the following table. These are utilized by multiplying the already calculated mg.% by the respective conversion factor.

Factors Used in the Marshall Determination

	UNKNOWN					
	Sulfanil-amide	Sulfa-pyridine	Sulfa-nilyl	Sulfa-thiazole	Sulfa-methyl	Sulfa-diazine
Sulfanilamide		1.47	2.07	1.48	1.54	1.50
Sulfapyridine	0.68		1.45	1.04	1.11	
Sulfanilyl-Sulfanilamide	0.48	0.69		.75	0.80	
Sulfathiazole	0.68	0.96	1.33		1.08	
Sulfamethyl-Thiazole	0.65	0.90	1.25	.92		

V. Modified Method of Hall and United States Army Laboratory for the Determination of Alcohol in Blood, Urine and Spinal Fluid.

A. Solutions:

1. Anstie's Reagent, modified, stronger.

Potassium dichromate (reagent quality) 3.70 grams
Distilled water . . . . 150 cc
Concentrated Sulfuric Acid (C.P. quality) . . . . 280 cc
Distilled water . . to make . . . . . . 500 cc

Dissolve the potassium dichromate in the 150 cc of distilled water and add slowly, with constant stirring, the sulfuric acid. Finally, dilute to 500 cc with distilled water. (Note: this solution is about 11% stronger than the ordinary modified Anstie's reagent, and should not be confused with that solution. It should be plainly labeled stronger Anstie's reagent. It may, however, be readily converted into modified Anstie's reagent by diluting 9 parts of the stronger Anstie's reagent with 1 part of distilled water, and may thus be used in carrying out the determination as given heretofore, if desired, instead of making up the two separate solutions).

2. Standard Alcohol Solution:

Absolute Ethyl Alcohol . . . . 2.53 cc
Distilled Water . . . . to make . . . . . 100 cc

Place about 50 cc of distilled water in a 100 cc volumetric flask and add the alcohol directly to the water to prevent its evaporation. The alcohol can be conveniently measured by means of a 1 cc serological pipette, transferring two full 1 cc proportions and finally the 0.53 cc portion.

3. Scott-Wilson Reagent

Mercuric cyanide . . . . 5 gm.
water .300 cc
Sodium Hydroxide . . . . 90 gm.
water . . . . .300 cc
Silver Nitrate. . . . . .1.45 gm.
water .200 cc

Add Sodium Hydroxide thoroughly cooled to Mercuric cyanide; mix thoroughly; then add Silver nitrate to the mixture with constant stirring. This solution will keep for six months. If it becomes cloudy or a precipitate forms, filter. Do not pipette this solution - it is very poisonous.

4. Preparation of Standards:

Arrange 9 test tubes of uniform diameter and color (color comparison tubes are better) in a test tube rack and place 9 cc of stronger Anstie's reagent (Solution) and distilled water in the amounts shown in the following table:

Tube No.	Alcohol Solution( )	Distilled Water	Corresponds to Alcohol in the Specimen
1	None	1.0 cc	Negative
2	0.1 cc	0.9 cc	0.5 milligrams per cubic centimeter
3	0.2 cc	0.8 cc	1.0 " " " "
4	0.3 cc	0.7 cc	1.5 " " " "
5	0.4 cc	0.6 cc	2.0 " " " "
6	0.5 cc	0.5 cc	2.5 " " " "
7	0.6 cc	0.4 cc	3.0 " " " "
8	0.7 cc	0.3 cc	3.5 " " " "
9	0.8 cc	0.2 cc	4.0 " " " "

The contents of each tube must then be thoroughly mixed. This may be accomplished by drawing the contents of each tube up into a 10 cc pipette and allowing it to run back into the tube several times.

If desired these standards may be kept for several weeks if tightly stoppered and kept in a vertical position in a test tube rack. The solutions in the standards must not come into contact with the stoppers, as both cork and rubber stoppers contain reducing substances which may cause a change in color of the standards.

Each standard should be labeled with the number of milligrams of alcohol to which it corresponds (e.g., tube No. 1 should be labeled "Negative"; tube No. 5 should be labeled "2.0 mg."; etc.).

It is useless to try to make standards for readings greater than 4.0 milligrams of alcohol per cubic centimeter, as the Anstie's reagent is apparently completely changed by that amount of alcohol, and no difference can be detected between the 4.0, the 4.5 and the 5.0 mg. per cc standards, no matter by which method they are prepared. Should specimens be encountered which have 4.0 mg. of alcohol per cc, or more, a second determination should be made using half the quantity of the specimen (2 cc instead of 4 cc) and the result multiplied by two to give the final reading.

B. Procedure: Urine, blood or spinal fluid:

Arrange two 27 x 210 mm. tubes with two-holed rubber stoppers and inlet and outlet tubes. The inlet tubes should extend nearly to the bottom of the tube and the outlet tube just below the stopper. Using well-washed rubber tubing, connect the inlets and outlets in such a manner that a current of air may be aspirated through the specimen tube over into the tube containing Anstie's reagent.

In the specimen tube place 4 cc of specimen, 2 to 4 cc of Scott-Wilson reagent, and sufficient water to make 10 cc. Half way between the upper level of the fluid contents and the bottom of the stopper, place a wad of glass wool.

In the second tube, place 10 cc of the Anstie's reagent.

Stopper the tubes, and adjust the suction so that a reasonable current of air is aspirated through the tubes. Immerse the tubes in a water bath previously brought to boiling. Continue the boiling and aspiration for 12 to 15 minutes.

Cool the dichromate solution and compare with the standards which read directly in milligrams per cc.

TABLES AND EQUIVALENTS

1 myriagram	=	10,000 grams
1 kilogram	=	1,000 grams
1 hectogram	=	100 grams
1 decagram	=	10 grams
1 gram	=	wt. of 1 cc of water
1 decigram	=	1/10 part of 1 gram = .1 gram
1 centigram	=	1/100 part of 1 gram = .01 gram
1 milligram	=	1/1000 part of 1 gram = .001 gram 1 mgm.

℥	=	fluid ounce
ʒ	=	fluid dram
O	=	pint
m	=	minim
cc	=	cubic centimeter

Min.		cc	Min.		
1	=	.06	60 m	=	1 ʒ
5	=	.30	8	=	1 ℥
10	=	.60	16	=	1 O
15	=	1. (.92)	32	=	1 qt.
20	=	1.25	2 O	=	1 qt.
30	=	1.90	4 qts.	=	1 gal.
1 fluid ʒ	=	4. (3.75)			
1 fluid ℥	=	30.00 (29.57)			
16 fluid ℥	=	473.17 = 1 pt.			
32 fluid ℥	=	946.33 = 1 qt.			

Grams		Grains
.0010	=	1/64
.0162	=	1/4
.0324	=	1/2
.065	=	1
.100	=	1 1/2
.500	=	7 1/2
1.	=	15
2.	=	30

		Grams
1 ℥	=	28.350
16 ℥	=	453.60 = 1 lb.

SUPPLEMENT TO BLOOD CHEMISTRY

VI. Non-Protein Nitrogen - all tubes in which Nessler's have been in must be rinsed with concentrated HNO_3.

5 cc. Blood filtrate (in pyrex tube); graduated at 35 and 50 cc.
1 cc. Diluted acid mixture (50 cc. of 5% Copper Sulfate
300 cc. of 85% Phosphorous Acid
100 cc. of Con. H_2SO_4)
(Dilute 1/2 of this solution with 1/2 of Distilled H_2O)

Boil vigorously with glass bead until tube fills with vapors, cover end of tube with funnel; continue heating 2 min., at least until solution is clear.

Cool 90 seconds
15 cc. to 20 cc. Distilled H_2O with tube at 45° angle
Cool to room temperature qs. to 35 cc.
15 cc. Nessler's Solution (stopper)
(Add simultaneous with standard)

Standard must contain 0.3 mg. of N per cc.
(.4716 Gm. Ammonium Sulfate qs. to liter)
3 cc. Ammonium Sulfate standard in 100 cc. Vol. flask
2 cc. Dil. phosphoric sulfuric acid mixture
60 cc. Dist. H_2O.
30 cc. Nessler's Solution
qs. to 100 cc. with Dist. H_2O.

Calculation:

$$\frac{RS}{RU} \times 30 = \text{mg. }\%$$

or

Place unknown at 30 in colorimeter, standard then = NPN per 100 cc.

Significance:

Normal NPN of blood is 25 to 35 Mg. %. NPN is a mixture of urea, uric acid, creatinine, ammonia, amine acids and a mixtur of other undetermined compounds.
Raised in terminal nephritis;
Lowered in uremia; urea determination more important.

VII. Blood Urea

Unknown - 5 cc. Blood Filtrate in 25 cc. Vol. flask
15 cc. Dist. H_2O
2.5 cc. Nessler's
qs. to 25 cc. with Dist. H_2O.

Standard- 3 cc. Standard Ammonium Sulfate in 100 cc. Vol. flask (10 cc. = 1 mg. N)
60 cc. Dist. H_2O
10 cc. Nessler's Sol.
qs. to 100 cc. with Dist. H_2O

Calculation:

$\frac{RS}{RU} \times 15$ = mg. %

Signigicance:

Normal urea of blood is 10 to 15 mg. %. Early nephritis may rise to 30 to 40 mg. %. In uremia the urea is retained. High readings may be found in:

Bichloride poisoning
Double polycystic kidneys
Intestinal obstruction
Prostatic obstruction
Lead poisonings
Certain infections
Cardiac failure

The amount of urea gives an indication of surgical risk.

VIII. Creatinine

Unknown - 10 cc. free filtrate
5 cc. alkaline picrate solution
(15 cc. saturated picric acid solution)
(3 cc. 10% NaOH Sol.)

Standard - 5 cc. standard (5 cc. =.03 mg.)

(In 500 cc. Vol. flask 3 cc. of the standard Creatinine Solution add 50 cc. of .1N to HCl, dilute to 500 cc. with Dist. H_2O)
(Standard Creatinine Solution =.1 Gm. of Creatinine in 100 cc. of .1N-HCl)
15 cc. Dist. H_2O
10 cc. Alkaline Picrate Solution (see above)

Stand 15 minutes

Calculation:

$$\frac{RS}{RU} \times 1.5 = \text{mg. \%}$$

Significance:

Creatinine is the least variable nitrogenous constituent of the blood. Normal 1 to 2 mg. %. Early nephritis, 2 to 4 mg. %. Chronic nephritis and uremia 4 to 35 mg. %, marked impaired kidney function, 4 to 5 mg. %. Anything over 5 mg. % is an unfavorable prognosis.

IX. Chlorides

10 cc. Blood Filtrate
10 cc. Dist. H_2O
10 cc. Silver Nitrate Solution (1 cc. = 1 mg. NaCl)
(2.905 Gm. Silver Nitrate in Dist. H_2O, then qs. to 1000 cc.) The Silver Nitrate and HNO_3 are not to be added simultaneously.
5 cc. Con. HNO_3

Stand in dark 5 minutes

.3 Gm. Powdered Ferric Ammonium Sulfate
Titrate with Standard Thiocyanate Solution (until definite salmon-red - not yellow

(1.7 Gm. KCNS qs. to 1000 cc.)
or
(1.4 Gms. NH_4CNS qs. to 1000 cc.)

Calculation:

cc. of $AgNO_3$ minus cc. of Thiocyanate Solution x by 100 = mg. %.

Example: 10 cc. NaCl - 5.5 cc. Thiocyanate = 4.5 x 100 = 450 mg. %.

Significance:

Normal-450 to 500 mg. % in whole blood
570 to 620 mg. % in plasma

Chiefly obtained from the condiment salt in food. About 5 Gm. is needed daily; excreted chiefly by kidneys, some by skin and intestines. When chlorides are retained, water is also retained in the body; decreased when body fluids are lost. CO_2 on exposure to air; chlorides go from cells to plasma. Acid into blood, as in acidosis, chlorides go from plasma to cells.

X. Plasma Proteins

A. Functions of blood.

1. To carry food from intestine and O_2 from lungs to tissue cells.
2. To carry waste products from tissue cells to excretory organs: kidneys, lungs, intestine, skin.
3. To carry hormones.
4. To aid in the defense of the body against disease.
5. To aid in the maintenance of H7, H_2O, temperature and all other equilibriums in the tissues.

B. Plasma: contains 9% solid material. Of this 9%, 7.5% is protein - most important constituents of blood plasma. Constituents are:

1. Fibrinogen
2. Euglobulin
3. Pseudoglobulin
4. Albumin
5. Nucleoprotein
6. Seromucoid

Fibrinogen is concerned with clotting; responsible for Sp. Gravity viscosity;colloid osmotic pressure, which has the important part to do with regulating the water exchanges between the blood stream and the body tissues.

Euglobulin and Pseudoglobulin have to do with the reaction in immunology only.

XI. Calcium in Blood

Normal-9 to 11.5 mg. %
Slightly higher in children than in adults; decrease in late months of pregnancy. After parathyroidectomy, calcium falls to a low level. At 3.5 to 7 mg. % will cause tetany. In severe nephritis, calcium will fall to 7. mg. %. Red blood cells have no calcium.

XII. Icterus Index

Centrifuge blood, pipette off serum
1 cc. of serum in large test tube
Pour few cc. of Standard Dichromate Solution in another tube (1:10,000)
Dilute serum with .9% Saline to about same depth of color
Note amount of saline used.
Dilution will be 1 cc. of serum plus number of cc. of .9% saline.

Calculation:

$$\frac{RS}{RU} \times \text{Dil. of Unknown} = \text{Icterus Index}$$

Significance:

Normal - 4 to 6

Above normal occurs in disease of the liver or biliary tract; also Hemolytic diseases such as Hemolyticanemia, Pernicious anemia; low values are found in secondary anemia.

LABORATORY TECHNICIANS MANUAL

PART IV

C O N T E N T S

SECTION I

GENERAL INTRODUCTION
THE PROTOZOA

PARASITOLOGY

General

A parasite may be defined as an animal or plant that lives within or upon another organism (the host) at whose expense it obtains some advantage without compensation. Broadly speaking, parasitology includes the bacteria, spirochetes, filterable viruses and fungi, as well as the protozoa, helminths and arthropods. However, it is customary to consider in this field only animal parasites which infect or infest the host, or serve as transmitters of pathogenic organisms.

Animal parasites are common in all countries, but are most numerous in the tropics. Some parasites cause serious disturbances in the host while others may give rise to no symptoms or apparent damage even though present in large numbers. Thus we see that the presence of a parasite does not necessarily imply pathogenicity. We are interested in non-pathogenic parasites only insofar as it is necessary to distinguish them from pathogenic species.

Parasitoses that are due to protozoa or helminths are known as infections, while those due to arthropoda are termed infestations. In certain infections man is the only host. In others there may be two or more hosts in which the host either matures or passes part of its life cycle. The host harboring the parasite during its sexual stages of development is called the definitive host. The intermediate host harbors the parasite during its larval or intermediate stages.

The signs and symptoms produced in man by pathogenic animal parasites are many and variable as to degree, and although the presence of a parasite may be suspected, a definite diagnosis can be made only through identification of the causative agent or its products from the body excreta, fluids or tissues.

Classification of Animal Parasites

Animals that are alike in all respects are classed together as a species. The male and female of a given species may be very unlike, but through mating they produce young that have characteristics similar to the parents. The term genus is of wider application and may include one or more species, being made up of animals that are similar in general structure. Genera that have certain characteristics in common make up a family. Families are grouped into orders, orders into classes and, finally, several classes may make up a phylum, the largest classification unit, of which there are several in the animal kingdom. In some cases there may be a further breaking down of these groupings into sub-classes, super-families, sub-families, tribes, etc.

In naming a species we always write the genus name first, commencing with a capital letter, followed by the species name which begins with a small letter. In some cases it will be noted that an author's name and date will follow, thus: Ascaris lumbricoides Linnaeus, 1758. The phyla of medical importance are: Protozoa, Platyhelminthes, Nemathelminthes and Arthropoda.

PHYLUM - PROTOZOA

CLASS	ORDER (OMITTED)	GENUS	SPECIES
Sarcodina (Rhizopoda) Move by means of pseudopodia		Endamoeba	E. histolytica
			E. coli
			E. gingivalis
		Endolimax	E. nana
		Iodamoeba	I. butschlii
		Dientamoeba	D. fragilis
Flagellata (Mastigophora) Move by means of undulating membrane or flagella		Trypanosoma	(T. gambiense
			(T. rhodesiense
			(T. cruzi
		Leishmania	(L. donovani
			(L. infantum
			(L. braziliensis
			(L. tropica
		Trichomonas	T. hominis
			T. vaginalis
		Chilomastix	C. mesnili
		Embadomonas	E. intestinalis
		Enteromonas	E. hominis
		Giardia	G. lamblia
Infusoria (Ciliata) Move by means of numerous fine cilia which are shorter than flagella. Have contractile vacuoles		Balantidium	B. coli
		Nyctotherus	N. faba
Sporozoa No motor organs. Parasitic in cells or tissues. Reproduce by spores		Eimeria	E. stiedae
		Isospora	I. hominis
		Plasmodium	(P. vivax
			(P. malariae
			(P. falciparum
			(P. ovale
		Sarcocystis	S. tenella

The Protozoa are the simplest forms of animals, being composed of a single cell. Protozoal cells are made up of protoplasm which is divided into nucleus and cytoplasm. In some instances the cytoplasm may be separated into an outer hyaline portion, the ectoplasm and an inner granular portion, the endoplasm. The ectoplasm is concerned with protecting the organism and with the procurement of food, excretion and sensation. It also gives origin to the structures responsible for locomotion. The type of motor organ (organelle) serves as the main basis for classifying protozoa. Types of organelles are: pseudopodia, flagella, undulating membranes, cilia.

The endoplasm is concerned with growth and reproduction. Contained in the endoplasm is the nucleus which is necessary to reproduction and the maintenance of life. In many protozoa it consists simply of a chromatin mass without definite structure. In others, some or all of the following parts may be observed: nuclear membrane; chromatin granules; karyosome; linin network; centrosome. Additional structures seen in the endoplasm of the protozoa include: contractile vacuoles which expand and contract at regular intervals; food vacuoles; chromatoidal bodies; ingested materials such as food particles, bacteria, etc.

In the flagellates, besides the nucleus, there may be a secondary nucleus, the kinetoplast which is composed of two parts: the parabasal body and the blepharoplast. The flagellum may arise from the latter. The portion of the flagellum immediately arising from the blepharoplast is called the axoneme.

Food is obtained with the aid of the organs of locomotion and may be ingested through the ectoplasm or through a rudimentary mouth (cytostome). Respiration is accomplished by absorption of oxygen and elimination of carbon dioxide through the ectoplasm, (aerobic), or by breaking down complex substances in the endoplasm (anaerobic). Excretion takes place in one of the following ways: by diffusion through the ectoplasm; by expulsion from vacuoles; during reproductive activities.

Substances secreted by protozoa include ferments, enzymes, toxins and pigments. The ferments are active in digestion, and the other substances may in some cases be responsible for damage done to the host. Some organisms also secrete a substance which hardens to form a protective coating or cyst. Encystment serves to protect the organism from adverse conditions. In some instances reproduction take place during the encysted state.

Reproduction may be sexual or asexual. A simple division of the organism into two parts is called fission. When it divides into many parts, each with separate cytoplasm and nucleus, we have schizogony. In sexual reproduction there is usually an alternation of generations with one phase of the life cycle being completed in each of two different hosts; sexual in one, asexual in the other.

Class - Sarcodina
(The Amoeba)

Endamoeba histolytica

This parasite is world wide in distribution but is more common in the tropics and in regions where sanitation is poor. On the basis of surveys it is estimated that 5% to 10% of the people in the United States harbor this organism. Infection with Endamoeba histolytica is known as Amebiasis or Amebic Dysentery. This is the most important protozoan parasite found in the intestine of man. The portal of entry and primary site of infection are the mucous membrane of the lower small intestine and the mucosa of the entire large intestine. Extension to the liver and other organs may occur, resulting in amebic abscess.

Infection takes place by ingestion of food or drink containing cysts. The cysts pass through the stomach unchanged, but through the action of intestinal secretions the cyst wall becomes permeable and four motile amoeba emerge. With the aid of a cytolytic substance which they secrete, they penetrate the intestinal mucous membrane where they multiply and give rise to characteristic "bottleneck" ulcers of variable size and extent.

Symptoms produced by this invasion are extremely variable but are, in general, proportionate to the degree of ulceration. There may be alternating periods of diarrhea and constipation. In a severe case there will be an excessive number of bowel movements attended by abdominal pain with a progressive weakening of the patient. Bowel discharges may consist almost entirely of blood and mucus with shreds of mucous membrane.

When an active diarrhea exists, organisms that are moved out do not have time to become encysted. One should, therefore, look for motile trophozoites in diarrheal stools. Since the type of motility is important in identifying the trophozoite, stool examination must be done within 20 to 30 minutes after passage, keeping the specimen warm, otherwise motility may be lost.

When the diarrhea ceases and stools become formed, the organism then has time to become encysted. Cysts are very resistant to adverse conditions, and there is, therefore, no immediate hurry in examining formed stools.

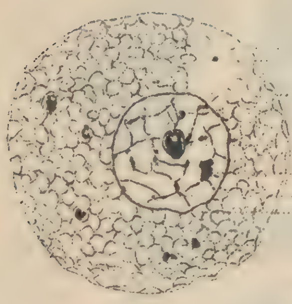
The laboratory diagnosis of Endamoeba histolytica is justified if amoeba are found in the feces which show the following characteristics:

Trophozoites

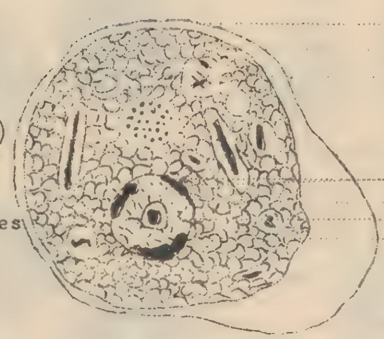
1. Active, progressive motility, directional in character.
2. Hyaline, finger-shaped pseudopodia.
3. Ingested red blood corpuscles.

Life Cycle of Eimeria perforans in the intestinal epilhelium of a Rabbit

1-7 Schizogong
8-13. Macrogametogony
14. Oocyst
15-19. Microgametogony



Metazoan Cell



Protozoan Cell

GRAPHIC DIFFERENTIATION OF AMOEBA

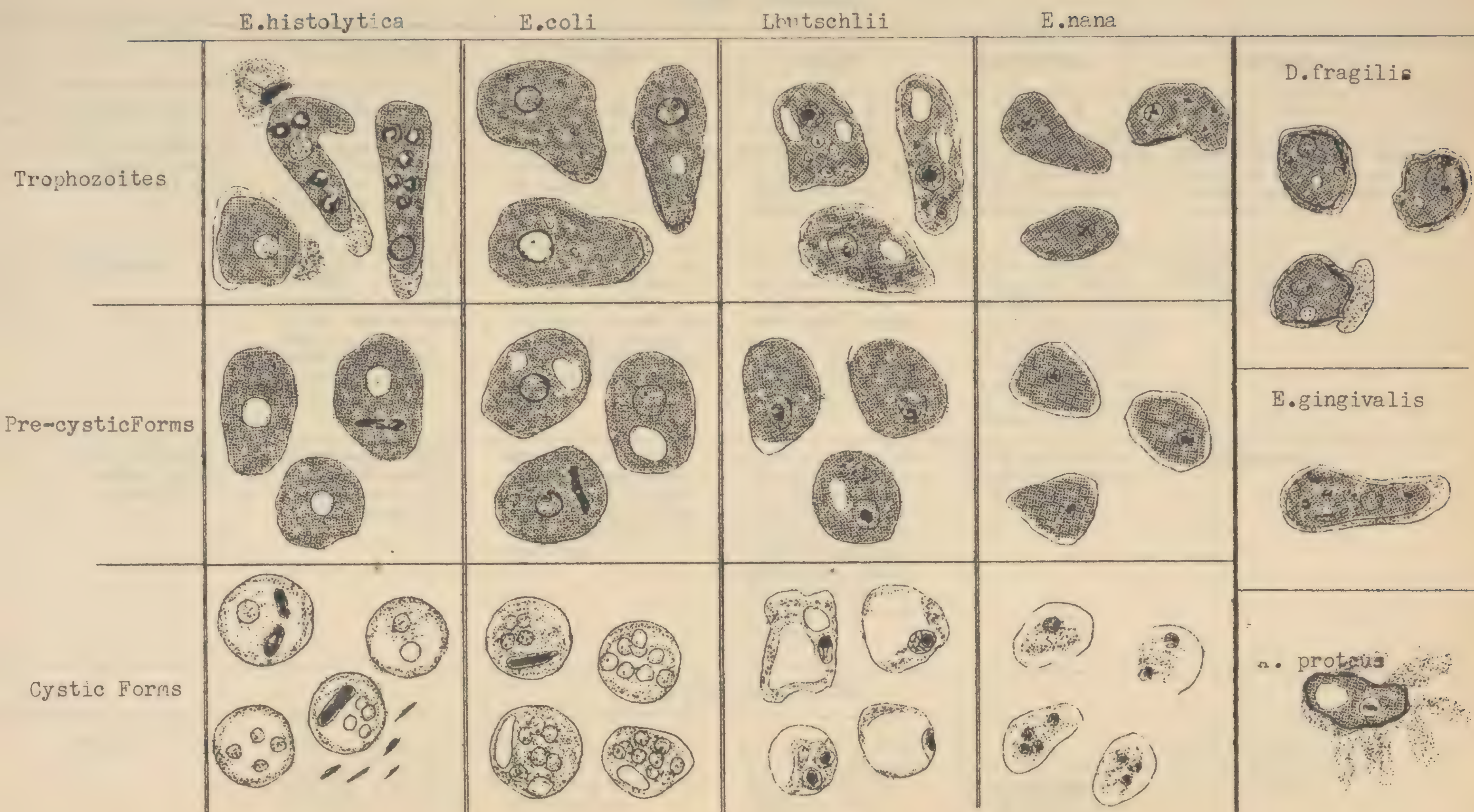


TABLE 86--CHARACTERISTICS OF THE AMOEBAE OF MAN

STAINED TROPHOZOITES

	E. HISTOLYTICA	E. COLI	E. NANA	I. BUTSCHLII	D. FRAGILIA
Average Size	20 to 35 u	15 to 30 u	6 to 10 u	9 to 13 u	3 to 12 u
Nuclear membrane (stains faintly or not at all)	Lined with minute, fairly even sized grains of chromatin which stain deeply.	Lined with coarse irregularly sized grains or bars of chromatin which stain deeply.	Chromatin on nuclear membrane in thin line and stains poorly.	A few poorly staining, widely separated, chromatin grains on nuclear membrane.	Chromatin on nuclear membrane in thin line and stains poorly. Nucleus frequently double.
Karyosome	Short rod or globule of small diameter, centrally suspended within the nucleus. Regular outline. Stains deeply and uniformly.	Short rod or ball or irregular outline, usually eccentric. Diameter greater than that of E. Histolytica. Stains deeply and uniformly.	Very large, central or eccentric, composed of 1, 2 or more deeply staining masses in a lighter staining matrix. Outline often irregular and oblong.	Similar to that of E. Nana but larger and more apt to contain a poorly staining central portion. Causes the nucleus to appear like an eye with a widely dilated pupil.	Composed of several minute deeply staining, discrete grains.
Linin network (stains faintly or not at all)	Contains no chromatin grains between the karyosome and nuclear membrane.	Sometimes contains grains of chromatin. Region just without karyosome halo often appears cloudy after staining.	It is not often discernible. Consists of a few short lines from the karyosome halo to the nuclear membrane. (Karyosome usually the only structure visible in the nucleus.)	Like a web when defined by an excellent stain.	Not demonstrable

THE LIVING TROPHOZOITES

	E. HISTOLYTICA	E. COLI	E. NANA	I. BUTSCHLII	D. FRAGILIA
Motion	Active progression in a definite direction. Form is elongated in motion.	Most stains are not actively progressive but merely change in conformation.	Some organisms like that of E. Histolytica (except the amoeba is very small) but the majority merely change in form and do not move progressively.	Many stains in culture like that of E. Histolytica. The majority, however, are like E. coli.	Slow indefinite progression or merely change in conformation.

	E. HISTOLYTICA	E. COLI	E. NANA	I. BUTSCHLII	D. FRAGILIA
Pseudopodia	Finger-like with smooth outline when not in progressive motion, Ectoplasm is clear, glass-like and easily discernible, When in progressive motion the ectoplasm may not be clearly differentiated. One-third to one-quarter of the parasite is ectoplasm.	Usually blunt, but it may be like E. Histolytica. The ectoplasm is usually not clearly differentiated. One-quarter to one-fifth of the parasite is ectoplasm but it is often poorly differentiated from the endoplasm even when the amoeba is in motion.	Like E. Histolytica. One half to one-third of the parasite is ectoplasm and is easily differentiated.	Like E. Histolytica or very broad with coarsely indented outline. One-half to one-third of the parasite is ectoplasm and is easily differentiated.	Often comprises one-half of the organism. Outline is often indented.
Color	Faint Green	Gray	Gray	Faint Green	Gray
Visibility of neucleus (oil-immersion lens)	Usually difficult to visualize except when the nucleus passes into the pseudopodia and is contrasted against the clear ectoplasm.	Quite clear. It is much more readily seen than that of E. Histolytica.	The karyosome may be defined with ease.	The karyosome may be defined with ease.	Difficult to distinguish from ingested bacteria.
Endoplasmic inclusions of diagnostic significance.	Red blood cells are typical and diagnostic. Degenerated and culture forms contain bacteria.	A voracious feeder but usually does not ingest red blood cells. Bacteria and starch grains are the principal inclusions.	Bacteria	Bacteria	Bacteria
			CYSTIC STAGE		
Average Size	5 to 20 u	10 to 30 u	5 to 8 u	7 to 15 u	
Nuclei number	1 to 4, rarely more. Mature cysts contain 4 nuclei.	1 to 8, rarely more Mature cysts contain 8 nuclei.	1 to 4, rarely more Mature cysts contain 4 nuclei.	1 or 2	
Visibility of nuclei in the unstained living state.	Poor but discernible with the oil-immersion lens.	Good	Good	Good	

Average Size	5 to 20 u	10 to 30 u	5 to 8 u	7 to 15 u
Shape	Generally spherical or nearly so.	Generally longer than broad and one side may be less curved than the other.	Irregularity of shape is common. Generally oval.	Great irregularity of shape and outline is common.
Reserve food inclusions (these disappear in old specimens and are not constant in young cysts.)	Bar-shaped chromatoid bodies in 0-90 per cent of cysts. Sometimes a small amount of glycogen is present in young cysts. It is diffuse and stains a light brown with iodine.	Acicular chromatoid bodies present in about 10 per cent of cysts. A large amount of glycogen may be present and push the nucleus against the cyst wall.	Small granules or masses of volutin and glycogen may be present. Neither is characteristic.	Masses, grains of rods of volutin may be present but these are not characteristic. The glycogen, almost invariably present, is large in amount, smoothly outlined and stains a deep brown with iodine. This iodine body is characteristic and diagnostic.

[1] Cystic stage of D. fragilis is unknown.

Cysts

1. Nuclei 1 to 4 in number.
2. Minute, centrally located karyosomes.
3. Large chromatoidal masses with rounded ends.

Blastocystis hominis, a vegetable organism should not be mistaken for the cyst of E. histolytica. It is spheroidal and from 5 to 30 microns in diameter. It consists of a central vacuole surrounded by a rim of cytoplasm containing many nuclei which stain black with hematoxylin.

The symptoms and signs produced in acute Amebic Dysentery are very similar to those in Bacillary Dysentery. Although laboratory identification of the causative organism is necessary for a definite diagnosis, presumptive evidence may be gained by an examination of the fecal exudate. In the case of Amebic Dysentery it will be found that the exudate shows few pus cells with a relatively high bacteria content, while in Bacillary Dysentery about 90% of the exudate consists of pus cells, and the bacteria content is usually low.

Methods to be used for laboratory examination are:

1. Microscopic examination of

   a. Fresh and stained feces.
   b. Material from amebic abscesses.
   c. Stained tissue sections.

2. Cultivation.

3. Complement fixation.

Non-Pathogenic Intestinal Amoeba

Two other common, but non-pathogenic amoeba, of worldwide distribution, are E. coli and E. nana. Life cycles of both are similar to that of E. histolytica and they are transmitted in the same manner.

I. butschlii and D. fragilis are apparently of wide distribution, but are least commonly found of the non-pathogenic amoeba. (For chief differential features of intestinal amoeba, see adjoining table).

Class - Ciliata

Balantidium coli

This ciliate is the cause of Balantidic Dysentery, a chronic affection of the large intestine, resembling Amebic Dysentery, but usually less severe. It is commonly found in the intestine of pigs and apes, but is of relatively rare occurrence in man. Infection takes place through ingesting food or drink contaminated with feces containing cysts of B. coli.

B. coli is of widespread distribution, human infection having been observed in many countries of Europe, the Philippines, China, Africa, Central and South America, and in many of the United States.

Laboratory diagnosis depends upon finding the organism in the feces. Methods used for detection are the same as for intestinal amoeba. The trophozoite can usually be found and is too large to be overlooked. It is an actively moving oval organism, 50 to 100 microns in length and 40 to 70 microns in width. It is covered with cilia which are in constant motion, and has a funnel-shaped mouth (cytostome) at the anterior end. Included in the endoplasm are: a large bean-shaped macronucleus and nearby a smaller round micronucleus; food vacuoles; two contractile vacuoles, which pulsate at regular intervals - a large one anteriorly and a smaller one posteriorly.

Class - Flagellata

Intestinal and Vaginal Flagellates

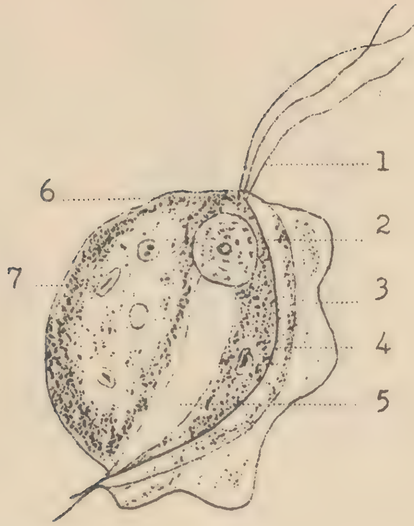
The common intestinal flagellates are Chilomastix mesnili, Giardia lamblia and Trichomonas hominis. With the exception of T. hominis they exist in both vegetative and encysted state. No intermediate host is necessary and it is doubtful whether the human species are found in animals. These parasites are commonly regarded as non-pathogenic, although they may at times be associated with diarrhea. They are classified according to the number of flagella and whether or not they have an undulating membrane and blepharoplast.

T. vaginalis is a common parasite of the female vagina where it may give rise to a catarrhal type of inflammation with leucorrhea.

Methods for laboratory examination are essentially the same as for the amoeba. The flagella may be demonstrated by staining a slide preparation with a drop of iodine and examining under darkfield illumination. The chief diagnostic features of each will be found in table adjoining.

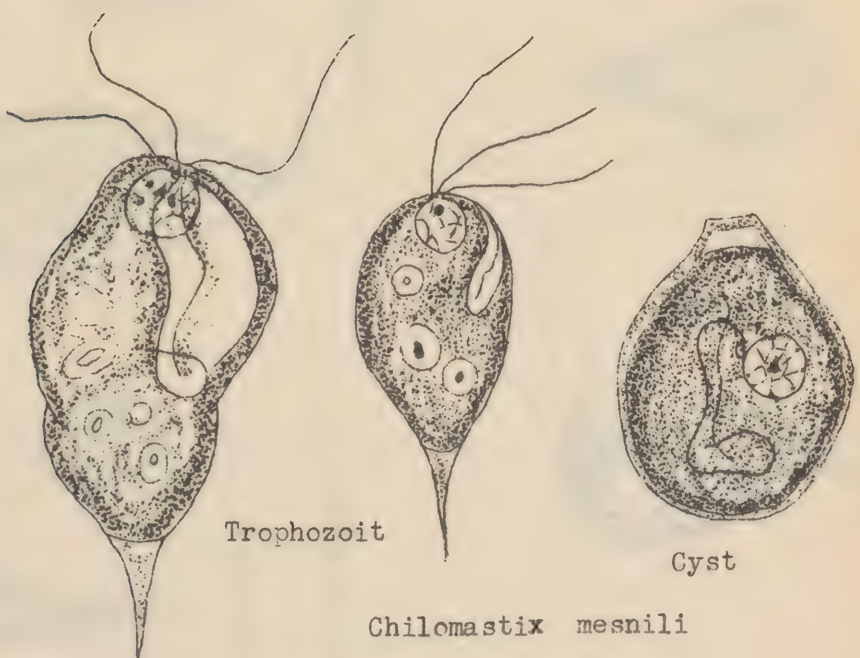
1. Peristome.
2. Cytostome.
3. Cytopharynx.
4. Food Vacuole.
5. Contractile Vacuole.
6. Micronucleus.
7. Macronucleus.
8. Cytopyge.
9. Cilia.

Balantidium coli



<u>Trichomas</u> <u>hominis</u> (Davaine 1860)

1. Flagella.
2. Nucleus.
3. Undulating Membrane.
4. Basal fibre.
5. Axostyle.
6. Blepharoplast.
7. Food Vacuole.

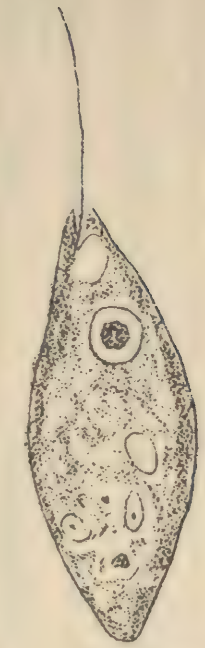


Chilomastix mesnili

Trophozoite

Cyst

Bodo caudatus



Trophozoite

Trophozoite

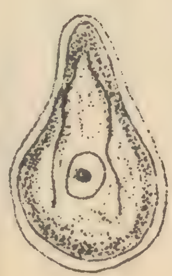
Copromonas subtilis

Lateral View
Trophozoite

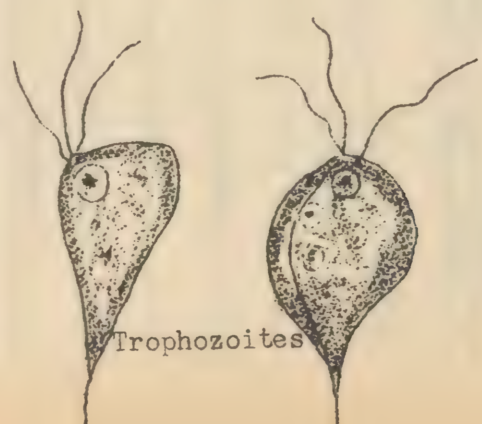
Cyst.

Giardia lamblia
1. Crossing anterior flagella
2. Blepharoplast.
3. Nucleus.
4. Sucker
5. Axostyle.
6. Parabasal body.

Trophozoite



Cyst



Trophozoites

Cysts

DIFFERENTIAL FEATURES OF SOME OF THE COMMON INTESTINAL AND VAGINAL FLAGELLATES OF MAN

	CHILOMASTIX MESNILI	GIARDIA LAMBLIA	TRICHOMONAS VAGINALIS	TRICHOMONAS HOMINIS
Shape	Elongated Pear	Pear-shaped	Pear-shaped	Pear-shaped
Size	10 to 15 Micra	12 to 15 Micra	15 to 18 Micra	10 to 15 Micra
Flagella	4-3 anterior 1 in cytostome	8-4 anterior, 2 caudal, 2 ventral	5-4 anterior, 1 posterior	5-3 to 5 anterior, 1 posterior
Undulating Membrane	None	None	Present	Present
Spiral Groove	Present	None	None	None
Sucking Disk	None	Present	None	None
Mouth Cavity	Present	None	Present	Present
Nucleus	1	2	1	1
Cyst	Yes	Yes	No	No
Cyst-shape	Lemon-shaped	Oval	- - -	- - -
Size of Cyst	7 to 9 Micra	9 to 12 Micra	- - -	- - -
Cyst - No. of Nuclei	1	4	- - -	- - -
Motion	Jerky, progressive	Jerky, progressive	Progressive	Progressive
Specimen	Feces	Feces, bile	Vaginal swab	Feces
Pathogenicity	None	Questionable	Questionable	None

Class - Flagellata (Cont'd)

Blood and Tissue Flagellates

Family - Trypanosomidae. This family is divided into six genera, two of which will be considered: Trypanosoma and Leishmania.

Genus - Leishmania

Members of this genus have only leptomonas and leishmania forms in their life cycle. (See adjoining figure). They have both vertebrate and invertebrate hosts. There are three recognized species parasitic in man, morphologically identical. In man a typical leishman donovan body appears ovoidal, 2 to 3 microns in longest diameter and contains a nucleus and kinetoplast. These organisms are usually enclosed in monocytes, polymorphonuclear leukocytes or endothelial cells. With Wright's Stain the cytoplasm is pale blue and encloses a rather large red nucleus. The cell membrane may be indefinite. The kinetoplast stains red and appears as a rod lying at right angles to the nucleus. In the invertebrate host and in cultures, the ovoidal body becomes spindle-shaped, and a single flagellum arises from the kinetoplast at the anterior end. Reproduction is by longitudinal binary fission for all members of the family Trypanosomidae.

1. Leishmania donovani

This species is the cause of Kala Azar or Dumdum Fever, a disease characterized by enlargement of the spleen and liver; long, irregular fever; anemia, with progressive loss of weight and strength. Approximately 90% of untreated cases end fatally in two to three years. With early treatment a majority of cases can be cured. Transmission is thought to take place through the sand fly. Possibly some cases are transmitted by the oral route.

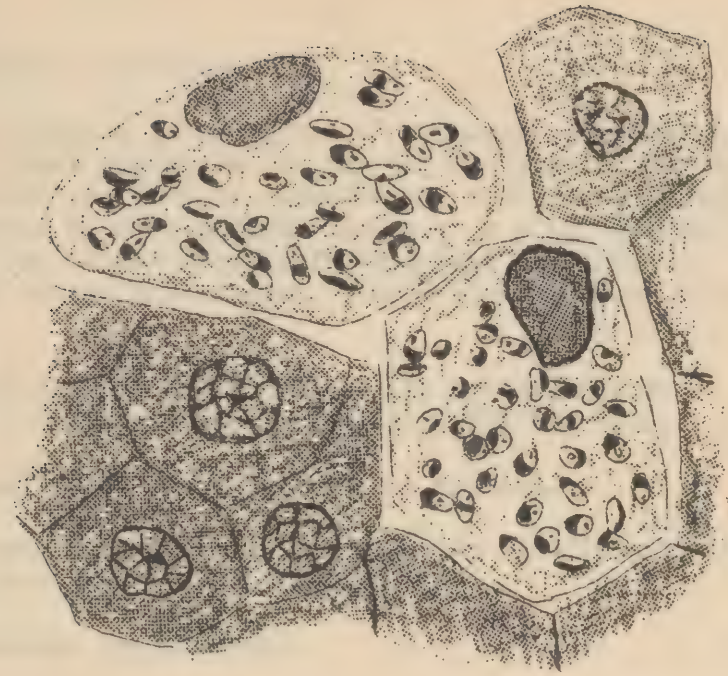
Distribution: Eastern India, North China, countries bodering the Mediterranean, the Sudan, West Africa, Iraq, Southern Russia.

Laboratory Diagnosis: the presence of the parasite must be demonstrated.

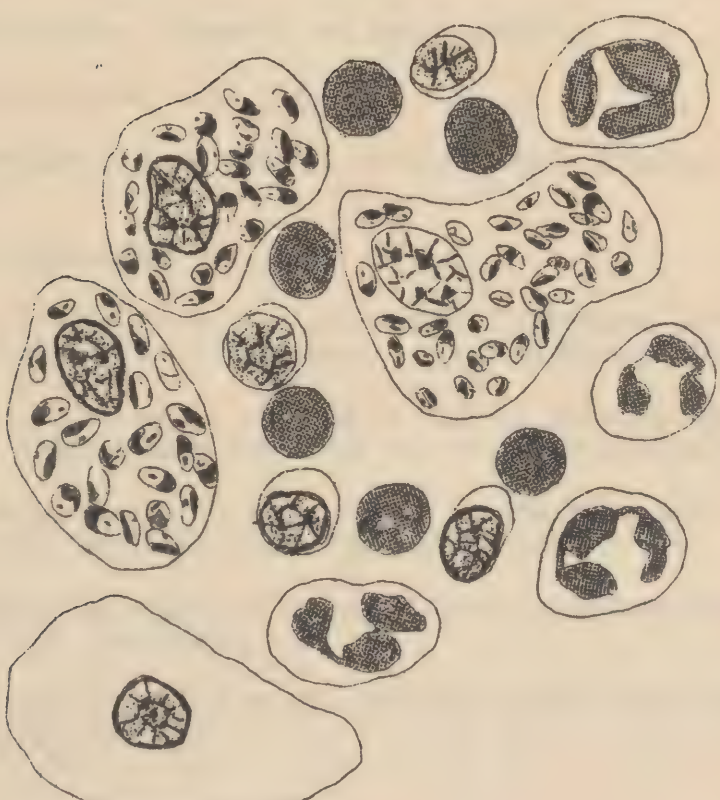
a. Smears may be made from the blood, liver, spleen, lymph glands, bone marrow and stained with Wright's Stain for microscopic examination.

b. Blood culture

c. Presumptive Tests (Globulin Precipitation
(Aldehyde Test
(Antimony Test



Leishmania donovani in the Kupfer Cells of the Liver



Leishuania tropica in Smear from Cutaneous Lesion

2. Leishmania tropica

This organism is the cause of cutaneous leishmaniasis (Oriental Sore). Ulcers may appear on any exposed part of the body. They are usually about one inch in diameter and have rounded edges. Healing may take place in from several months to a year, leaving a disfiguring scar. One attack results in immunity. L. tropica apparently does not invade the viscera. Uncomplicated cases never end fatally.

Distribution: India, Persia, Palestine, Africa, countries around the Mediterranean, Costa Rica, Brazil, Peru.

Transmission: sand fly, direct contact.

Laboratory Diagnosis: find the parasite in material from edge of ulcer. Culture may also be used.

3. Leishmania braziliensis

Infection with this species results in muco-cutaneous leishmaniasis, an ulcerating infection similar to Oriental Sore, except that the lesion tends to spread to the mucous membranes of the mouth, nose and throat, and may result in deformities, loss of voice, etc.

Distribution: Central and South America.

Transmission: Sand fly.

Laboratory diagnosis: As for Leishmania tropica.

Genus - Trypanosoma

Members of this genus have leishmania, leptomonas, crithida and trypanosoma stages in their life cycle. (See adjoining diagram)

An insect as well as a vertebrate host is probably necessary for each species. Transmission may be in one of the following ways:

Through the mouth parts of the intermediate host while feeding on blood of the definitive host;

Through ingestion of infected feces of intermediate host by vertebrate;

By infected feces of insect being rubbed into wound made by bite.

A typical trypanosome stained with Wright's appears as a spindle-shaped body, 15 to 30 microns or more in length with a delicate reddish membrane arranged into folds running along one side. The cytoplasm stains pale blue and may contain dark blue volutin granules. The nucleus lies near the middle and stains reddish purple. The kinetoplast is dark red and lies at the posterior end. The single flagellum arises at the posterior end (blepharoplast), runs along the edge of the undulating membrane and terminates as a free flagellum at the anterior end.

In fresh blood preparations, trypanosomes are colorless and through their rapid movement, may give a spinning motion to red blood corpuscles.

Apparently all trypanosomes are harmless to their invertebrate hosts. Three species are of medical interest: T. gambiense, T. rhodesiense and T. cruzi.

1. Trypanosoma gambiense

Causes African sleeping sickness, a disease characterized in the early stages by irregular fever, lymph gland enlargement, anemia and weakness. In the later or sleeping stage, nervous and mental symptoms appear, the victim becomes emaciated, is apathetic and may sleep most of the time. Treatment is effective in a large percentage, if given early. After the appearance of the sleeping stage, most cases progress to coma and death within a few years.

2. Trypanosoma rhodesiense

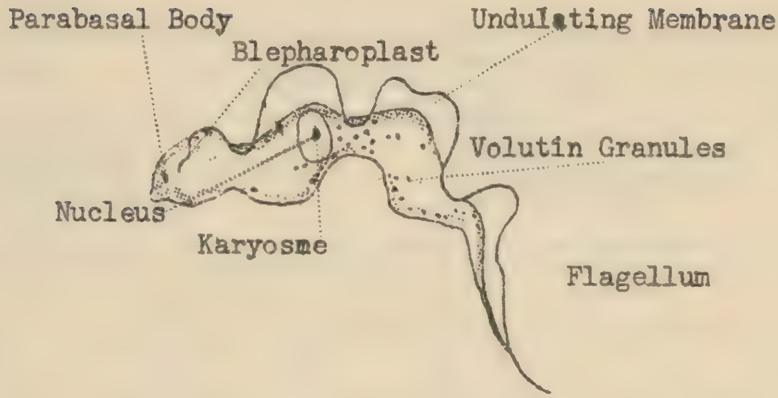
Is the cause of a more virulent form of African sleeping sickness. The disturbances caused are similar to those listed for T. gambiense, but the disease usually ends fatally within one year unless early treatment is given.

T. gambiense and T. rhodesiense are similar in appearance. Laboratory differentiation is based upon finding posterior nucleated forms in laboratory animals that have been inoculated with the trypanosomes. Apparently a larger percentage of T. rhodesiense will develop the posterior nucleated forms.

3. Trypanosoma cruzi

This parasite is the cause of Chaga's Disease or South American Trypanosomiasis. It lives in the blood as a typical trypanosome, in the tissue cells of man as a leishmania and in the intestinal tract of certain insects as a crithidia. In man the parasites are located in the reticulo-endothelial cells of the spleen, liver and lymphatic tissues, and in the cells of the heart muscles.

Diagram of a Trypanosome

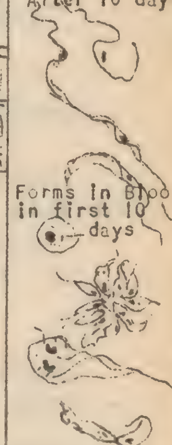


Forms Found in Life of Trypanosomes

1. Leischmania. 3. Crithidia

2. Leptomonas. 4. Trypanosoma.

DIFFERENTIAL CHART OF TRYPANOSOMES OF MEDICAL IMPORTANCE

Disease Produced	African Sleeping Sickness of Man.	African sleeping sickness of man Virulent form.	Chagas disease - South American. Trypanisomiasis of Man.	Trypanosomiasis of Rats Non-pathogenic of Man.
Culture	Culture Media - NNN plus glucose 32°C: Ponselles' medium at 25°C. Cultivation difficult	Same as for T. gambiense	Culture Media NNN 25°C.	Culture Media NNN. 25°C.
Susceptible Lab-Animals	All except monkeys.	All except monkeys.	Guinea pigs, bats, mice, rabbits, dogs, cats, monkeys.	White Rats, Mice with difficulty
Invertebrate Host (Vectors)	Tsetse Fly, G. marsitans, G. palpalis, G. brevipalis, G. pallidipes, G. tachinoids (Bite infective after 20-30 day cyclic development)	Same as for T. gambiense	Kissing Bug, T. megista, T. infestans, T. sordida, Rhodnius prolixus, possibly Cimex lectularius.	Fleas. (C. canis, Pulex irritans, etc)
Vertebrate Host	Man and probably Antelope	Man and probably Antelope	Man. Armadillo, probably Cat, O'possum	Rats
Leishmania Forms in Tissue or Blood	No	No	In Tissue	In Blood
Dividing Forms in Peri. Blood	Yes	Yes	Never	Yes
Drawings from Blood Smears and Tissue	Forms in Blood 	 Parasites same as T. gambiense except in laboratory animals in which posterior nuclear forms develop.	 Forms in Blood	After 10 days 
Parabasal Body. Size & Shape	Small. Inconspicuous	Small. Inconspicuous	Small. Inconspicuous Conspicuous, Large and Oval	Large and Rod-shaped
Position of Nucleus	Middle 1/3	Middle 1/3 and sometimes Posterior 1/3.	Middle 1/3	Anterior 1/3
Type	Polymorphic (forms without and with flagella in blood stream)	Polymorphic	Monomorphic	Monomorphic
Length	13 to 40	18 to 35	16 to 24	20 to 30
Geographic Distribution	Tropical Africa	East Africa (Rhodesia, Nyasaland, Tanganyika, Mozambique)	South America and probably Central America	Cosmopolitan
Species	T. gambiense	T. rhodiense (probably a virulent strain of T. gambiense	T. cruzi	T. lewisi

Chaga's Disease is found in acute and chronic forms. The acute stage usually lasts 20 to 30 days and is characterized by fever, swelling of the face, enlargement of the thyroid and lymphatic glands, and of the liver and spleen. If the victim survives, the disease may become chronic and last for many years, giving rise to a variety of symptoms.

During the acute stage the trypanosomes may be found in the peripheral blood. In the chronic form the trypanosomes disappear from the blood, but the leishmania forms are present in the tissues.

For additional information on these parasites, see Differential Chart of Trypanosomes. Methods used for laboratory diagnosis are outlined in Section III.

Class - Sporozoa

Genus - Plasmodium (Malaria Parasites)

P. vivax	(tertian)
P. malariae	(quartan)
P. falciparum	(malignant tertian, Estivo-Autumnal)
P. ovale	(mild, similar to tertian)

Malaria is a group of closely related infections due to protozoan parasites that live chiefly in the blood, and are transmitted by Anopheline mosquitoes. Three common species of the genus plasmodium are associated with malarial fever in man: Plasmodium vivax, P. malariae and P. falciparum. A fourth species, similar to P. vivax, has been identified. Since this parasite (P. ovale) is comparatively rare, it will not be described here.

This infection typically is characterized by severe paroxysms of chills, fever and sweating, which may occur at regular intervals corresponding to the period of time necessary for the asexual cycle of development. The paroxysms may occur daily (quotidian), on alternate days (tertian) or at 3-day intervals (quartan). However, in many instances, these symptoms are neither regular in occurrence nor clearly defined. Frequently the only symptom noted is an irregular fever. Anemia of varying degree occurs in all types of malaria, but is usually most pronounced with P. falciparum infections. Uncomplicated infections with P. vivax, P. malariae and P. ovale rarely end fatally - most malaria deaths being due to P. falciparum. Cerebral malaria and Blackwater Fever may also occur with infections caused by P. falciparum.

In nature, malaria is transmitted only by the bite of the female Anopheles mosquito. Experimentally, the infection may be transferred by injecting blood of an infected person into another. More than 120

species of Anopheline mosquitoes have been identified. About 20 species of this group carry malaria in different parts of the world. The chief carrier in southern United States is Anopheles quadrimaculatus. On the Pacific coast, in Canada and Europe, the main carrier is A. maculipennis.

Malaria is one of the most prevalent of all preventable diseases; it is the scourge of the tropics. It has a wide geographic distribution, occurring from the Arctic Circle to the Equator, but is more prevalent and more virulent in warm, moist climates. The Tertian form has the widest distribution, being common in temperate regions, as well as in the tropics. Quartan malaria is least commonly found of the three main types, except in Central and West Africa. Estivo-Autumnal malaria is the prevailing type in most tropical regions and is generally confined to hot, moist climates.

Repeated infections with malaria leave a pronounced resistance. It is well known that mosquitoes do not bother some individuals - possibly on account of body odor. Negroes are much more resistant to Tertian malaria than whites, indicating an immunity to this particular plasmodium.

The Tertian parasites may remain latent in the spleen or other organs for several years. Exposure, overwork, fatigue, etc., may then cause an attack. In badly infected regions, as high as 60-70% of the population may be carriers.

There are two cycles in the life history of the malaria plasmodia: (1) the asexual cycle in Man (Schizogony); (2) the sexual cycle in certain species of female Anopheles mosquitoes (Sporogony). All of the known species infecting man go through the same developmental stages, but with the exception of P. vivax and P. ovale, which are similar, different time periods are required.

The Asexual Cycle

Only P. vivax will be described. The differences in microscopic appearances of the other types will be found in Differential Table of Malaria Parasites.

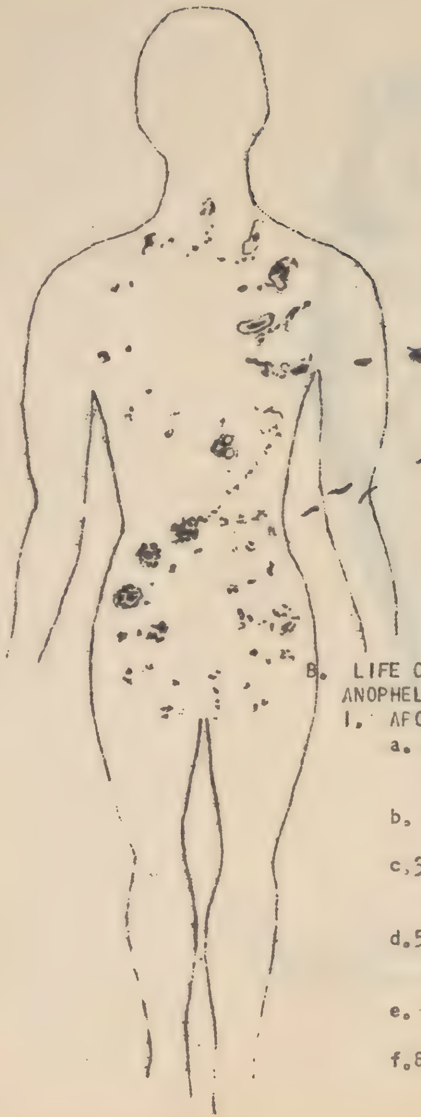
When the infected Anopheles female secures a blood meal, she injects saliva containing sporozoites into the wound. Apparently the sporozoites are carried by macrophages to the reticulo-endothelial system, where they reproduce by segmentation for the first 4 or 5 days after entering the body (exo-erythrocytic stage). About the 5th day

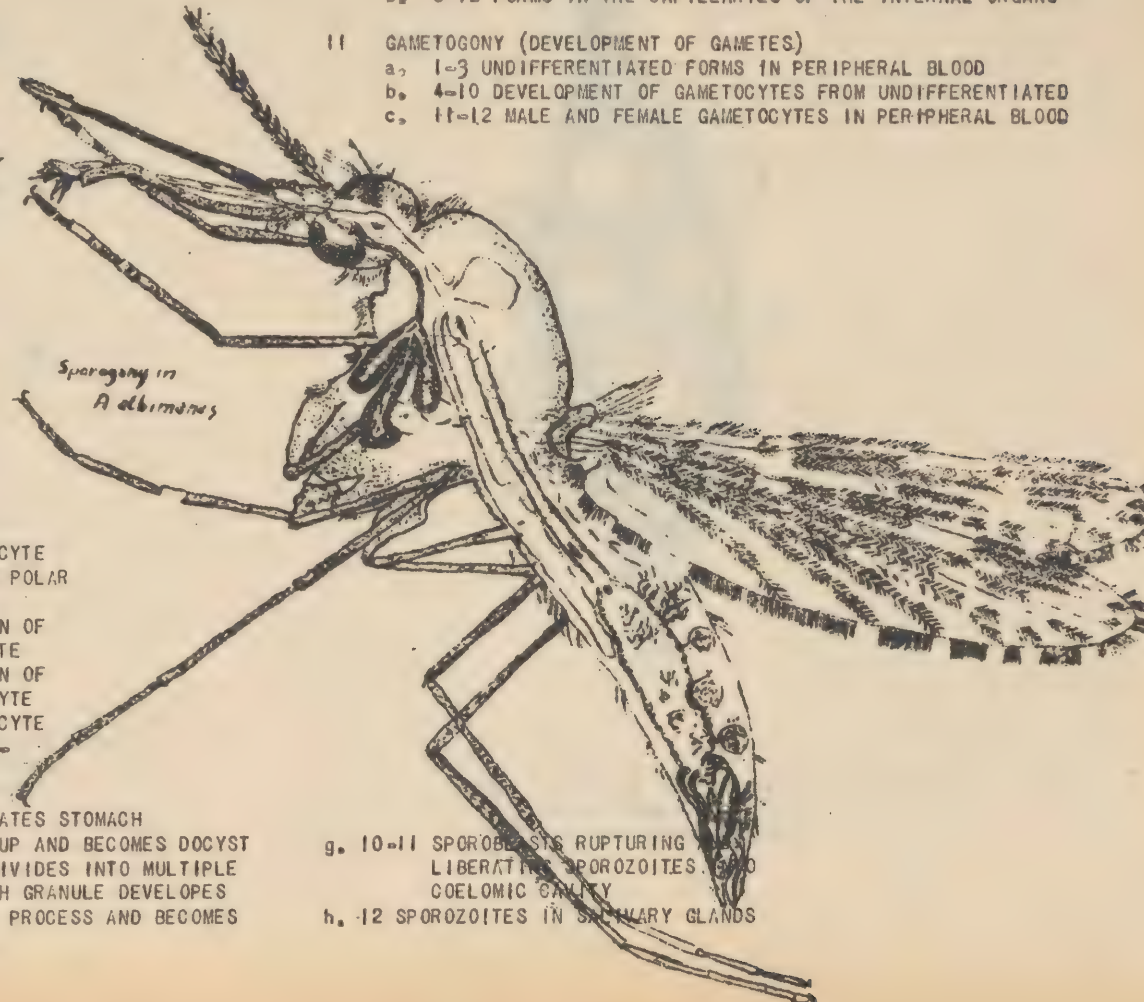
PLASMODIUM FALCIPARUM

LIFE CYCLE IN MAN AND IN FEMALE ANOPHELINE MOSQUITO

A. LIFE CYCLE IN MAN
  I. SCHIZOGONY (ASEXUAL CYCLE)
    a. 1-3 FORMS IN THE PERIPHERAL BLOOD
    b. 6-12 FORMS IN THE CAPILLARIES OF THE INTERNAL ORGANS

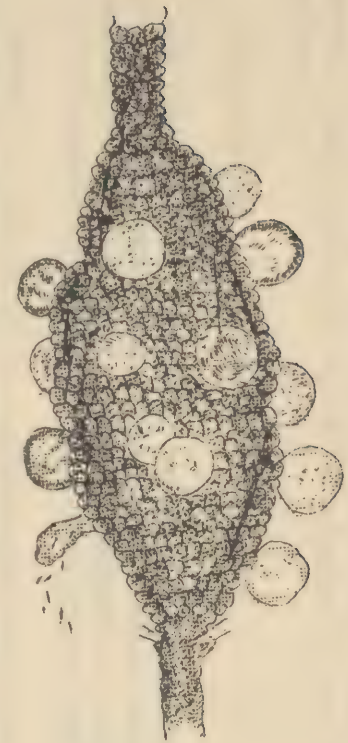
  II GAMETOGONY (DEVELOPMENT OF GAMETES)
    a. 1-3 UNDIFFERENTIATED FORMS IN PERIPHERAL BLOOD
    b. 4-10 DEVELOPMENT OF GAMETOCYTES FROM UNDIFFERENTIATED
    c. 11-12 MALE AND FEMALE GAMETOCYTES IN PERIPHERAL BLOOD





B. LIFE CYCLE IN FEMALE ANOPHELINE MOSQUITO
  I. AFOROGONY
    a. 1-FEMALE GAMETOCYTE WITH EXTRUDED POLAR BODIES
    b. 2-EXFLAGELLATION OF MALE GAMETOCYTE
    c. 3-4 FERTILIZATION OF FEMALE GAMTOCYTE BY MALE GAMTOCYTE
    d. 5-6 ZYGOTE OR MOTILE OOKINETES
    e. 7-ZYGOTE PENETRATES STOMACH WALL, ROUNDS UP AND BECOMES OOCYST
    f. 8-9 THE NUCLEUSDIVIDES INTO MULTIPLE GRANULES. EACH GRANULE DEVELOPES A CYTOPLASMIC PROCESS AND BECOMES A SPOROZOITE.
    g. 10-11 SPOROBLASTS RUPTURING AND LIBERATING SPOROZOITES INTO COELOMIC CAVITY
    h. 12 SPOROZOITES IN SALIVARY GLANDS

Portion of Mosquito Salivary Gland Showing
Infection with Malarial Sporozoites
(Oil Immersion magnification)



Mosquito Stomach Showing Malarial Oocysts In
Various Stages of Development
(low power magnification)

they invade red blood corpuscles, and proceed to grow and segment, destroying the infected erythrocytes in the process. Forty-eight hours are required from the entrance into the erythrocyte until the bursting forth of daughter parasites (merozoites) in the case of P. vivax. Liberated merozoites re-enter red cells to repeat this cycle, and after about two weeks from the time of infection (incubation period) enough parasites have been produced to cause symptoms. When regular bouts of chills and fever occur, the time of chill corresponds to the rupture of parasitized red cells. All stages of development may be seen in the blood at one time, but usually one form predominates. The various recognizable stages of development are listed below.

Trophozoites

In its earliest form, the parasite appears as a small hyaline ring in the infected erythrocyte. In about 6 hours ameboid activity may be seen in fresh preparations. With Wright's or Giemsa's stain the ring form shows a blue ring of cytoplasm with a small red nucleus.

Growth of the parasite continues and the infected red cell becomes pale and swollen. With good staining, fine pink dots (Schuffner's) will usually be seen in the infected erythrocyte, and small yellowish brown pigment granules are present in the cytoplasm of the parasite. At the end of about 36 hours two-thirds of the red cell is filled by the parasite, ameboid motion is lost and the pigment granules collect near the center.

Schizont

After about 40 hours the parasite fills the cell and its chromatin usually divides into 16 to 24 irregular masses distributed throughout the cytoplasm.

Merozoites

Just before segmentation, the cytoplasm of the parasite divides into equal sections, one about each segment of chromatin. At the end of 48 hours the mature merozoites rupture out of the red cell and each one then seeks to enter a new erythrocyte to repeat the cycle. The pigment is liberated into the blood stream to be destroyed by phagocytes. Stained mature merozoites, before liberation appear as a collection of oval or round blue bodies, each having a bright red or purple chromatin dot near the periphery.

Gametocytes

Some of the liberated merozoites which enter red cells do not go through the asexual cycle of segmentation as outlined, but gradually enlarge to become sexual forms (gametocytes). The reason for this difference in development is unknown, but it probably represents an effort of the parasite to overcome increasing resistance of the host. These are the forms responsible for initiating the sexual cycle in the mosquito. It takes 4 days for the development of the gametocytes from ring form to maturity, and they must be 7 to 10 days old before they are infectious to the mosquito. Stained male forms (microgametocytes) are 7 to 8 microns in diameter and do not completely fill the enlarged red cell. The cytoplasm appears pale blue and contains scattered brown pigment granules. The nucleus stains red, is diffuse and located near the center of the parasite. The female form (macrogametocyte) is larger, filling the red cell; the cytoplasm is darker blue and the nucleus is compact and not centrally located. There are usually more female than male forms.

The Sexual Cycle

When the female Anopheles takes a blood meal from an infected human host, all forms of the parasite may be ingested. Upon reaching the mosquitoes' stomach, the male forms send out 8 to 10 flagellar-like processes, each containing nuclear material. These break off (microgametes) and each seeks out a matured female (now macrogamete), which is fertilized. The resulting organism is called the zygote. This becomes motile (ookinete), and moves into the stomach wall where encystment takes place (oocyst). The chromatin in the cyst undergoes division with the formation of as many as 10,000 granules in small clumps. Each chromatin granule becomes a cigar-shaped sporozoite. The cyst ruptures and motile sporozoites liberated into the body cavity make their way to the salivary glands. It requires 7 to 12 days for this cycle with favorable temperature and humidity. The mosquito apparently is not harmed by these parasites.

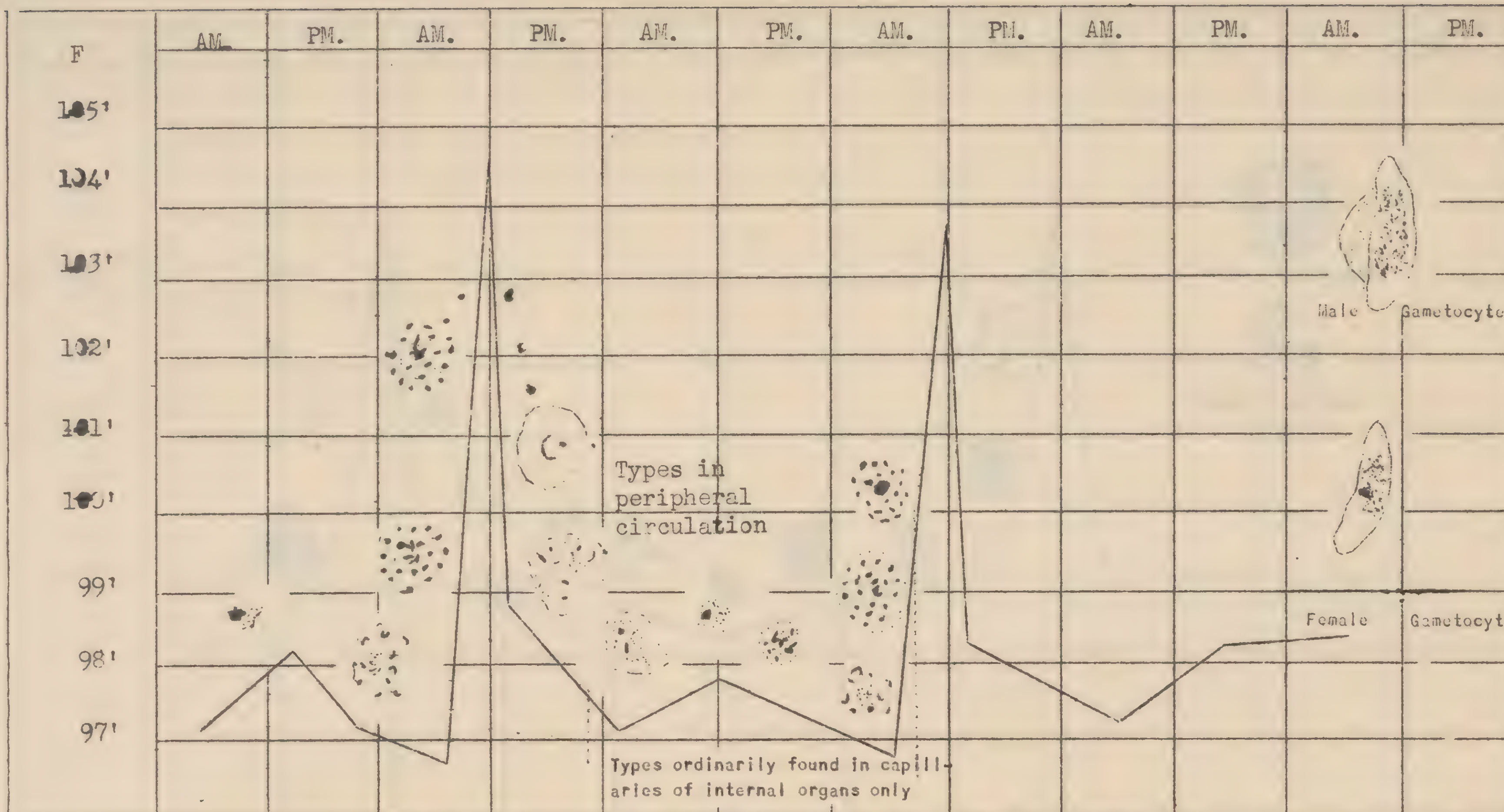
Laboratory Diagnosis

A definite diagnosis of malaria can only be made by finding the causative parasite in thick or thin blood films. Blood for examination should be taken during the period from 12 hours after a chill until 1 or 2 hours before the next one is expected. When only ring forms are found, additional blood films should be taken 8 or more hours later, in order to determine the species.

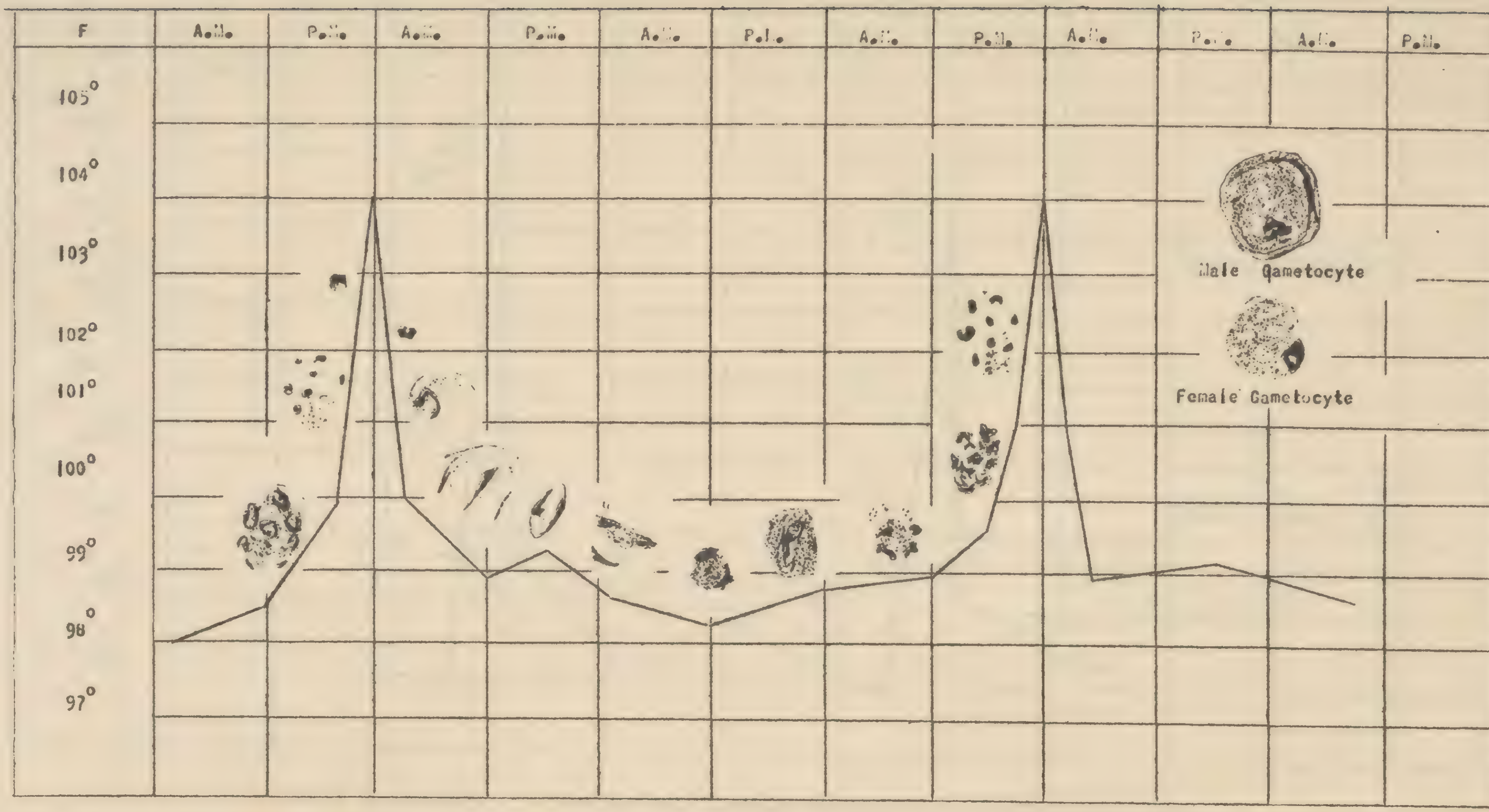
Laboratory diagnostic procedures are outlined in Section III.

DIFFERENTIAL TABLE OF MALARIAL PARASITES OF MAN

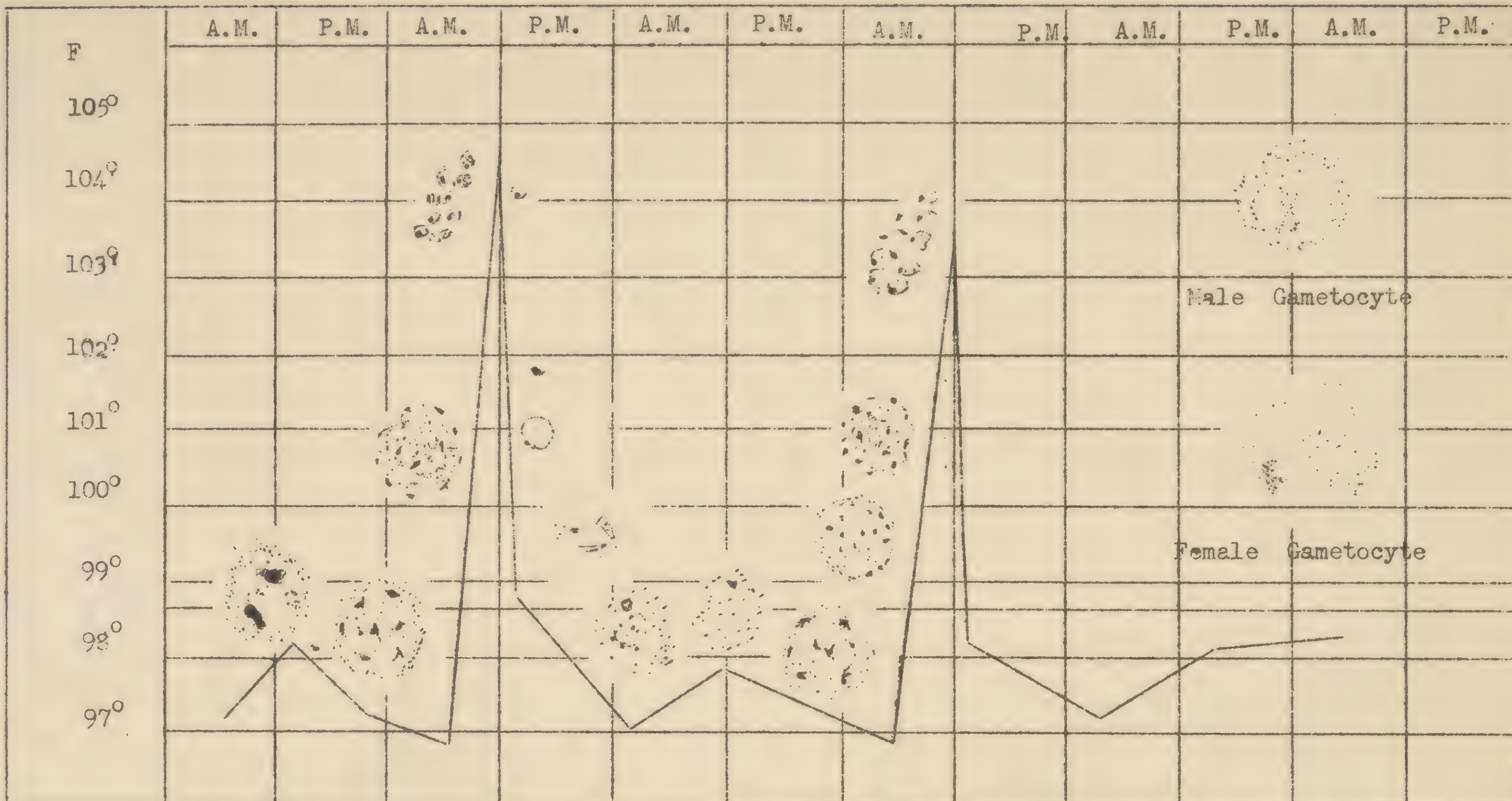
Total deaths (absolute numbers) U.S.Army 1920-1930 inclusive			2 (8.32%)		(4.16%)		21(87.62%)	
Total admissions (absolute numbers) U.S.Army 1920-1930 inclusive			10,714 (75.1%)		128 (0.9%)		3,432 (24%)	
Seasonal prevalence in sub-tropical regions where all 3 types of malaria are present			Spring and early Summer		Late Fall		Late Summer and Early Fall	
SPOROGONY	Time required to complete cycle at 20°C Relative Humidity 70		7 to 12 days		20 to 24 days		16 to 20 days	
GAMETOGONY	Length of Life Cycle		Four days. Infective for mosquitoes in 7 days.		Seven days. Infective for mosquitoes in 10 days.		Four days. Infective for mosquitoes in 7 days	
GAMETOGONY	Shape		♀♀ Round; ♂♂ Oval		♀♀ Round; ♂♂ Oval		Crescent shaped with male having ends more rounded	
GAMETOGONY	Size	♀	9μ to 10μ		6μ to 75μ		12μ x 2.5μ	
GAMETOGONY	Size	♂	7μ to 8μ		55μ to 65μ		12μ x 3μ	
SCHIZOGONY	Differential characters of schizonts		See illustrations		Band Forms. See illustrations		See illustrations	
SCHIZOGONY	PIGMENT	Color	Light Brown		Dark brown or black		Dark brown or black	
SCHIZOGONY	PIGMENT	Type	Fine Grains		Large amt. coarse grains		Coarse grains	
SCHIZOGONY	PIGMENT	Presence and Amt.	Yes. Large Amts.		Yes. Large amts.		Yes. Small amt.	
SCHIZOGONY	Stages present in peripheral blood at any given time		All stages, but one predominates		All stages, but one predominates		Ring forms & gametocytes only. Other forms in capillaries of internal organs except in severe cases	
SCHIZOGONY	Number of Merozoites formed		12 to 16		8 to 12		12 to 32	
SCHIZOGONY	Length of Life Cycle		48 hours		72 hours		48 hours	
SCHIZOGONY	Motility		Active ameboid		Sluggish ameboid		Little or none	
SCHIZOGONY	SIZE	Mature forms in parasitized R.B.C.	Fills cell		Fills cell		Fills only 2/3 cell	
SCHIZOGONY	SIZE	Ring forms first hour	1.5μ to 1.75μ		2μ to 2.5μ		0.5μ to 1.5μ	
PARASITIZED RED CELLS	Presence of Multiple Infection		Uncommon		Uncommon		Fairly common	
PARASITIZED RED CELLS	Stippling		Fine granules	Pink granules of Schuffner	Not present	A few basophilic gran.	Few coarse grains	Malignant stippling of Maurer stains
PARASITIZED RED CELLS	Color		Pale, yellowish Red	Pale, brownish red	Dull green	Greenish red or normal	Purpli Brassy Copper	Purplish Red or normal
PARASITIZED RED CELLS	Size		Enlarged to 10μ or 12μ		Not enlarged		Not enlarged	
SPECIES			Unstained	Stained	Unstained	Stained	Unstained	Stained
SPECIES			P. vivax (tertian or benign tertian.)		P. Malaria (quartan)		P. falciparum (aestivo-autumnal malignant tertian, subtertian or pernicious tertian)	



LIFE CYCLE OF P.FALCIPARUM IN MAN SHOWING RELATION TO TEMPERATURE CURVE.



Life Cycle of P. malaria in Man Showing Relation to Temperature Curve.



Life Cycle of P. vivax in Man Showing Relation to Temperature Curve.

SUMMARY TABLE

SHOWING
SOURCE OF MATERIAL FOR LABORATORY EXAMINATION AND
CHIEF DIAGNOSTIC CHARACTERISTICS OF PROTOZOAN PARASITES

SOURCE	PARASITE	CHIEF DIAGNOSTIC CHARACTERISTICS
BLOOD	Leishmania donovani	Oval, red-nucleated bodies in Reticulo-endothelial cells
	Plasmodium malariae	RBC not enlarged; rings, bands, ovals Schizonts 6-10 merozoites
	Plasmodium falciparum	Small rings, crescents, does not enlarge red cells
	Plasmodium vivax	RBC enlarged; pale, Schuffner's dots Schizonts 15-20 merozoites
	Trypanosoma gambiense	Active, slender trypanosome
	Trypanosoma rhodesiense	Active, slender trypanosome
	Trypanosoma cruzi	Short, stumpy trypanosome
INTESTINE	Balantidium coli	Large moving ciliate; bean-shaped macronucleus, globular micronucleus, contractile vacuoles
	Chilomastix	Flagellate; cytostome, spiral groove, lemon-shaped cyst
	Endamoeba coli	Trophozoite sluggish, no RBC, contains bacteria, mature cyst 8 nuclei
	Endamoeba histolytica	Trophozoite active, ingested RBC, no bacteria, mature cyst 4 nuclei

SOURCE	PARASITE	CHIEF DIAGNOSTIC CHARACTERISTICS
INTESTINE	Giardia lamblia	Pear-shaped flagellate, sucking disk, 2 nuclei, oval cyst
	Trichomonas hominis	Pear-shaped flagellate with undulating membrane
SKIN AND MUCOUS MEMBRANES	Leishmania donovani	Oval, red-nucleated bodies in Reticulo-endothelial cells
	Leishmania tropica	Oval, red-nucleated, intracellular bodies
BRAIN	Endamoeba histolytica	Trophozoite active, ingested RBC, no bacteria, mature cyst 4 nuclei
	Plasmodium falciparum	Small rings, crescents, does not enlarge red cell
	Trypanosoma cruzi	Short, stumpy trypanosome
	Trypanosoma gambiense	Active, slender trypanosome
	Trypanosoma rhodesiense	Active, slender trypanosome
MUSCLES AND TISSUES	Trypanosoma cruzi	Short, stumpy trypanosome
LYMPH NODES	Leishmania donovani	Oval, red-nucleated bodies in Reticulo-endothelial cells

SOURCE	PARASITE	CHIEF DIAGNOSTIC CHARACTERISTICS
LYMPH NODES	Trypanosoma gambiense	Active, slender trypanosome
	Trypanosoma rhodesiense	Active, slender trypanosome
SPLEEN	Leishmania donovani	Oval, red-nucleated bodies in Reticulo-endothelial cells
LIVER	Endamoeba histolytica	Trophozoite active, ingested RBC no bacteria, mature cyst 4 nuclei
	Leishmania donovani	Oval, red-nucleated bodies in Reticulo-endothelial cells

SECTION II

THE HELMINTHS

PHYLUM - PLATYHELMINTHES
(The Flatworms)

Class - Trematoda

Members of this class are commonly known as flukes. They are flat, unsegmented, leaf-shaped and have an alimentary tract but no anus. Most species have reproductive organs of both sexes in one parasite (hermaphroditic). The developmental cycle is a complicated one of alternation of generations, the intermediate host usually being a snail, mussel or crab. The ova are operculated in most, an important exception being the Schistosoma.

Human infection with the flukes is largely confined to the tropics and the Orient. Laboratory diagnosis depends upon identification of the ova in the appropriate body fluid or discharge.

Additional information regarding the common flukes of man will be found in Table on adjoining page.

Class - Cestoda

The cestodes or tapeworms are segmented, ribbon-shaped and have no alimentary tract. The adults consist of a series of flat, rectangular segments (proglottides) attached to a smaller segment, the head or scolex which is equipped to anchor onto the intestine of its host.

The segments arise from the scolex by budding. Each segment is hermaphroditic and in mature ones the uterus is filled with ova. The testes usually consist of a great number of small glands scattered throughout the segment. Ducts from these unite to form a single vas deferens leading to the genital pore which is found on one lateral border of the segment, or upon its flat surface. Sperm from the same or another segment reach the genital pore and are carried to the oviduct where the ova are fertilized. They then pass into the uterus where they may remain until the segment disintegrates or until passed out through a birth pore.

There are no circulatory, respiratory or digestive systems. Nourishment is obtained by absorption. Excretion takes place through four excretory canals which run the full length of the chain of segments and empty posteriorly. Near the lateral borders are two or more nerve cords derived from the scolex which also run the entire length.

As a rule the tapeworms pass a larval or encysted stage in the tissues of an intermediate host, only the adult stage occurring in man. Exceptions to this are Taenia solium and Hymenolepis nana. In the case of T. solium the cystic stage may take place in man, favored

DIFFERENTIATION OF THE COMMON FLUKES OF MAN

	FASCIOLA HEPATICA	FASCIOLOPSIS BUSKI	CLONORCHIS SINENSIS	PARAGONIMUS WESTERMANII	SCHISTOSOMA HAEMATOBIUM	SCHISTOSOMA MANSONI	SCHISTOSOMA JAPONICUM
Common Name	Liver Fluke	Large Intestinal Fluke	Chinese Fluke	Lung Fluke	Blood Fluke	Blood Fluke	Blood Fluke
Adult lives:	Liver	Intestine	Bile ducts	Lung	Pelvic veins	Mesenteric Veins	Mesenteric Veins
Spread by:	Water plants	Water chestnut	Raw fish	Raw crab	Wading in infected water	Wading in infected water	Wading in infected water
Specimen	Feces	Feces	Feces, bile	Sputum	Urine	Feces	Feces
Ovum	140 by 80 Micra Operculated	130 by 80 Micra Operculated	30 by 15 Micra. Vase-shaped Operculated	90 by 55 Micra Operculated	150 by 60 Micra. No Operculum Terminal spine	150 by 60 Micra. No Operculum Lateral spine	80 by 60 Micra. No operculum. Rudimentary spine (small hook)

Helminths. Diagram of diagnostic material. A, egg of the whipworm (trichocephalus trichiurus); B, egg of the pinworm (Enterobius vermicularis); C, egg of the broad fish tapeworm (Diphyllobothrium latum); D, egg of the dwarf tapeworm (Hymenolepis nana); E, egg of the Chinese Liver Fluke (Clonorchis sinensis); F, G, H, fertilized, unfertilized, and decorticated eggs respectively of the large intestinal roundworm (Ascaris lumbricoides); l, egg of hookworm (Necator americanus or Ancylostoma duodenale); J, fore-part of rhabditoid larva of hookworm showing long buccal cavity. Compare with short buccal cavity of rhabditoid larva of Strongyloides stercoralis, K; L, egg of either the pork tapeworm (Taenia solium) or the beef tapeworm (T. saginata); M, N, gravid segments of the pork and beef tapeworms respectively, showing difference in the number of uterine branches - fewer than fifteen in the pork tapeworm; more than fifteen in the beef tapeworm; O, egg of hookworm showing fully developed embryo. Such eggs are commonly found in constipated stools of hookworm patients. P, egg of the Oriental lung fluke (Paragonimus westermani); Q, egg of the Oriental blood fluke (Schistosoma japonicum); R, egg of Manson's blood fluke (S. mansoni); S, egg of the vesical blood fluke (S. hematobium); T, egg of the sheep liverfluke (Fasciola hepatica) or the large intestinal fluke (Fasciolopsis buski). Approximate magnifications, E X600, M and N X3; all others X300. Compiled and modified from various sources.

tissues being the eye and brain (cysticercus cellulosae). Apparently H. nana requires no intermediate host. There is, therefore, danger of direct infection (oral) from handling feces containing the ova of either of these parasites.

The portal of entry for all known tapeworm infections, except ocular sparganosis, is through the mouth. Eating raw or incompletely cooked meat of the intermediate host, which is infected with encysted larvae, is the source of infection with T. saginata, T. solium and D. latum.

The important tapeworms of man belong to three genera: Taenia, Hymenolepis and Diphyllobothrium.

1. Taenia saginata

This, the beef tapeworm, is the common tapeworm of the United States, and is widely distributed over the world. The scolex is about the size of a large pin head and is surrounded by four sucking discs. The terminal segments which become detached and appear in the feces, are about 18 to 20 mm. long and 4 to 7 mm. wide. The length of the worm varies from several to 30 or more feet. Cattle provide the intermediate host, the eggs or segments on reaching the soil being swallowed by cattle when grazing or when drinking contaminated water. The larva is set free in the intestine of the cow and makes its way to the muscle or other tissue where it becomes encysted.

Man is infested by eating raw or improperly cooked meat containing the cysts. On entering the stomach the cyst wall is digested, the scolex is set free, becomes attached to the intestinal wall and grows to maturity in 2 to 3 months, and the cycle is repeated. Since the head or scolex is the ancestor of the worm, it is important to make certain that the head has been passed.

Detection of tapeworm scolex: following the vermifuge administered by physician, the stool is saved in a container together with the stool passed before administration of the drug, and taken to the laboratory. The entire quantity of feces is passed through a #20 sieve. The scolex is identified by examining a segment between two glass slides and holding it to the light.

Laboratory diagnosis will depend upon finding the segments or ova in the feces. The ova are spheric or ovoid, yellow to brown in color and have a thick radially striated shell. Vegetable cells (generally present) are often mistaken for these ova.

DIFFERENTIAL CHARACTERISTICS OF THE IMPORTANT TAPEWORMS OF MAN

	DIPHYLLOBOTHRIUM LATUM	HYMENOLEPIS NANA	TAENIA SOLIUM	TAENIA SAGINATA	TAENIA ECHINOCOCCUS
Frequency in U. S.	4	1	3	2	5
Length of Adult Worm	10-30 ft.	1/2 to 4 1/2 Cm.	6-12 ft.	12-30 ft.	.3 to .8 Cm.
Suckers	2 (grooves)	4	4	4	4
Hooks	None	Present	Present	None	Present
Number of Proglottids	3000-4000	Up to 200	800-1000	1000-2000	3
Mature Proglottid	Broader than long	Broader than long	Longer than broad	Longer than broad	Longer than broad
Uterus	Central rosette	Irregular sac-like	7-12 branches	15-30 branches	Loose coil in end proglottid
Genital Pore	Middle	Lateral on one side	Lateral, Alter-nate	Lateral, Alter-nate	Lateral alternate
Ova	70 by 45 Micra, Operculated	45 by 35 Micra, Globu-lar, 2 mem-branes, hooklets	35 by 35 Micra, Radially striated cortex, hooklets	35 by 35 Micra, Radially striated cortex hooklets	
Intermediate Host	Cyclops and fish	None	Hog, man	Cattle	Man, sheep
Stage in Man	Adult	Adult	Adult Larval	Adult	Larval

2. Taenia solium

The pork tapeworm is comparatively rare in this country. Its life cycle is similar to that of the beef tapeworm - the pig being the intermediate host. The ova of Taenia saginata and Taenia solium are so closely similar that they are practically indistinguishable. Best method of differentiating the two is to recover mature segments from the feces. These should be cleaned and relaxed by shaking in the physiological salt solution. Segments may then be pressed between two glass slides and held up for examination in front of a strong light. Count the number of uterine branches. (See Table of Differential Characteristics).

3. Diphyllobothrium latum

Infection with the fish tapeworm occurs only in those regions where the species of fresh water fish that provide the intermediate host for this parasite, are found. This infection is common in Japan and many countries of Europe. It has been reported, not infrequently, in the United States.

The ova are usually easily found in the feces where the worm is present. They are brown in color and filled with small spherules. The shell is thin and has a small hinged lid at one end. The lid may be demonstrated by making sufficient pressure on cover glass to force it open.

4. Hymenolepis nana

The dwarf tapeworm is 1 to 4.5 cen. in length. It is more common in tropical countries, but is found in northern latitudes and is probably the most common of all the tapeworms in the United States. Transmission is direct, no intermediate host being required.

Laboratory diagnosis must usually depend upon discovery of ova in the feces, since the worms themselves are partly disintegrated as a rule when they leave the body. The ova are colorless, semi-transparent, nearly spheric and contain an embryo surrounded by two distinct membranous walls, between which is a broad zone of gelatinous substance. Three pairs of parallel hooklets can usually be seen inside the inner membrane.

5. Taenia echinococcus (Echinococcus granulosus)

The dog is the normal definitive host for this parasite. Usual intermediate hosts are cattle, sheep and hogs, but man and other mammals may be infected. The infection in man is known as Hydatid Disease and occurs wherever man lives in such close association with dogs that the hands or food become contaminated with their feces.

It is common in Iceland, Central Europe, Africa, Arabia, South America and Australia. The infection is rarely found in man in the United States, but is common in swine in some parts of the country.

Larvae develop from ingested ova and become encysted in various organs of the body, particularly the liver and the lungs. The cysts eventually cause serious disturbances as a result of pressure and tissue destruction, and unless completely removed surgically, death usually occurs in a few years. Diagnosis depends upon: (1) finding free scolices in fluid obtained from a cyst, or (2) an intradermal reaction (Casoni's Test), which consists of injecting a small drop of cyst fluid intradermally, and noting the reaction obtained.

PHYLUM - NEMATHELMINTHES
(Roundworms)

Class - Nematoda

The nematodes are cylindric worms varying in length from 1 mm. to 80 mm. in different species. They are unsegmented and possess a tubelike alimentary canal which has a distinct esophagus near the mouth. All are covered with a cuticle of varying thickness, formed from the ectoderm, which is frequently ringed. Inside the ectoderm is the body cavity containing clear fluid within which the reproductive organs lie. The sexes are as a rule separate. The male can usually be recognized by its smaller size and by the curved posterior end which may have an umbrella-like expansion - the copulatory bursa. The genital opening of the female lies ventrally and may be located close to the mouth or near the tail.

Vascular and respiratory systems are lacking. The nervous system consists of a nerve ring around the esophagus, dorsal and ventral nerve cords, and circular bridges connecting the cords. The excretory system usually consists of two tubes which discharge near the head. A few nematodes are viviparous (wuchereria, trichinella), but in most cases the female deposits ova of characteristic appearance.

The life cycles vary with the different species. As a rule, the larvae are different from the adult. An intermediate host is necessary for only a few species, but usually a larval stage takes place before a new host is infected.

1. Ancylostoma duodenale - Old World type) Hookworm
   Necator americanus - New World type)

Hookworm infection (ancylostomiasis) is a chronic affection characterized by gastro-intestinal disturbances, anemia, mental and physical lethargy. It is common in tropical countries, but is also found in subtropical and even northern latitudes where unhygienic conditions exist. The hookworm inhabits the duodenum and upper part of the small intestine. Multiplication does not occur in the body. The severity of an infection is proportionate to the number of worms.

Ancylostoma duodenale is common in southern Europe and in Egypt, and is not infrequently found in America. Necator americanus is common in central and southern Africa and in subtropical America and southern United States. The principal point of differentiation between the two species is that the Necator is provided with two pairs of plates in the buccal cavity (mouth), while the Ancylostoma has two pairs of hooklike teeth. The Necator is smaller than Ancylostoma, the male being 7 to 9 mm. long, the female 9 to 11 mm.

DIFFERENTIATION OF IMPORTANT ROUND WORMS OF MAN

	ASCARIS LUMBRICOIDES	ANCYCLOSTOMA DUODENALE AND NECATOR AMERICANUS	ENTEROBIUS VERMICULARIS	STRONGYLOIDES STERCORALIS	TRICHINELLA SPIRALIS	TRICHURIS TRICHIURA	FILARIA BANCROFTI
Disease	Ascariasis	Hookworm disease	Pinworm infection	Strongyloidiasis	Trichinosis	Whip worm infection	Filariasis Elephantiasis
Geographical Distribution	Cosmopolitan	Sub-tropics and tropics	Cosmopolitan	Sub-tropics and tropics	Cosmopolitan	Cosmopolitan	Tropics
Location in Man	Intestine	Intestine	Intestine	Intestine	Intestine	Intestine	Adult-Lymphatics, Larva, Blood
Size (mm) Male	150-300	8-10	2-5	.7	1.5	30-45	40
Female	200-350	10-13	8-13	2.2	3-4	35-50	83
Definitive Host	Man, hog	Man	Man	Man	Man, Carnivorous mammals	Man, monkey, hog	Man
Ovum	Broadly ovoid 60 by 45 Micra, Mammillate covering. Brownish	Oval, blunted ends, 60 by 40 Micra. Thin shell, clear zone. Segmented cytoplasm. Transluscent	Oval, flattened on one side, 55 by 25 Micra. Thin shell. Contains embryo Transparent	Ova not found in stool. Living larva	None	Oval with plugs in both ends 50 by 25 Micra. Brownish	None
Infective Stage	Embryonated ovum	Filariform larva	Embryonated ovum	Filariform larva	Encysted larva	Embryonated ovum	Microfilaria carried by mosquito
Site of entry	Intestine	Skin	Intestine	Skin	Intestine	Intestine	Skin
Development	Indirect to intestine via intestine, lymph, blood, lungs, throat	Indirect to intestine via lymph, blood, lung, throat	Direct	Indirect to intestine via lymph, blood, lung, throat	Direct larva encyst in muscle	Direct	To lymph nodes via lymph stream

The life histories of the two are probably the same. The ova pass out with the feces and under favorable conditions of warmth and moisture, develop an embryo which hatches in a few days. The resulting larvae, after several developmental changes, are ready to infect a new host. The chief point of entry is through the skin of the feet. From the skin the larva migrates through the lymphatics, or blood, to the heart and lungs. It then pierces the alveoli of the lungs and passes into the bronchioles, bronchus, trachea and pharynx, and eventually reaches the intestine in which it develops into the adult.

The severe anemia produced when worms are present in large numbers is probably due to: abstraction of blood by the worms and hemorrhage from bleeding points left when they change position in the intestine; continued bleeding - presumably due to a secretion in the buccal glands that prevents coagulation; toxic secretion of parasites; secondary microbic infection.

The blood picture may show a red blood cell count of 2 to 3 million, 35-50% hemoglobin, a low color index, and 10-25% eosinophils. The laboratory diagnosis usually depends on finding ova in the feces, since adult worms are seldom seen except after a vermifuge. The ova are nearly always typical, showing a thin, but very distinct shell, a clear zone and a finely, granular, segmented protoplasm.

2. Ancylostoma braziliense

This is a parasite of dogs and cats, and occurs in North and South America, Ceylon, India, Siam and the Philippines. Human infection usually takes the form of "creeping eruption" - a painful and itching dermatitis. Infective larvae are able to penetrate the skin and burrow extensively through the subcutaneous tissue, but apparently do not progress farther into the body. The adult worms (in dogs) are slightly smaller than A. duodenale and can be distinguished by their characteristic teeth - a small median pair and an outer pair that are larger. The ova are not distinguishable from those of A. duodenale.

3. Strongyloides stercoralis

This parasite may be found in warm, moist regions throughout the world. It is considered to be harmless, or at worst, as causing a mild catarrhal enteritis. The infection is acquired by filariaform larvae penetrating the skin, or through their being ingested. Upon reaching the alimentary tract, the females inhabit the walls of the duodenum and jejunum. Their eggs hatch in the tissues and larvae are passed out in the feces. When an active diarrhea exists, the ova may be passed in the feces. They are similar to the hookworm ova in appearance.

Laboratory diagnosis depends upon finding the larvae. It should be borne in mind that hookworm ova may hatch in 24 to 48 hours after the feces has been passed. The following points will aid in differentiating the two larvae:

Hookworm		Strongyloides
Ova found in fresh feces	-	Larvae usually
Larvae have deep mouth cavity	-	Mouth cavity shallow
Genital anlage small, inconspicuous	-	Large, conspicuous

4. Ascaris lumbricoides (Roundworm)

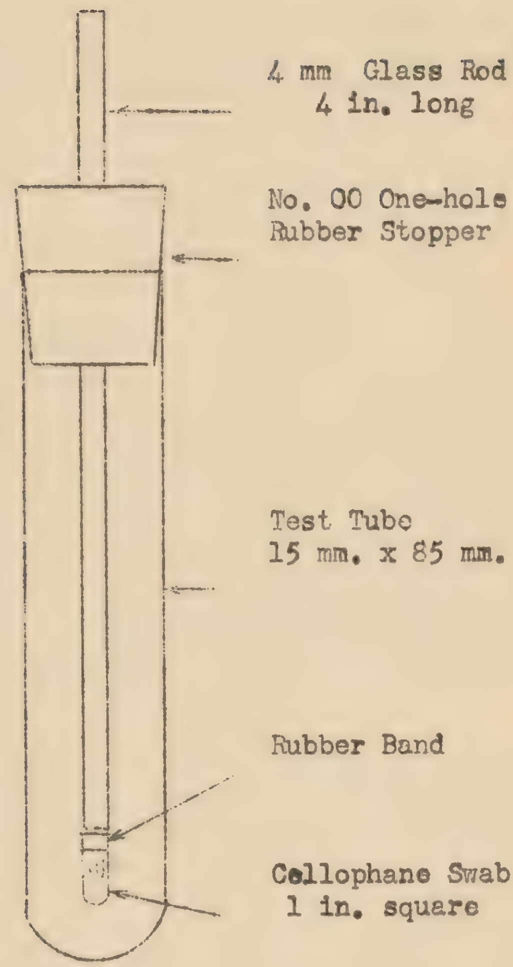
This parasite inhabits the small intestine and its presence is characterized by gastro-intestinal disturbances, malnutrition, anemia, cachexia and nervous symptoms. The male measures 15-20 cen. in length, and 3 mm. in diameter. The female is about twice as large as the male. It is the most common parasite of the intestine, especially in tropical countries. Found more frequently in children under 10 years of age, but occurs also in adults.

Eggs of the parasite which are in the feces are unsegmented, oval in shape and provided with a rough, irregular protective shell. Under suitable conditions, the eggs develop into an embryo and larva in the soil or water in about 2 weeks. The egg does not hatch until it enters the intestine of man or some susceptible animal. The larva is set free in the host and attains adult stage in 4 to 6 weeks, after which the eggs are discharged and the cycle repeated. Man is infected by drinking polluted water, eating contaminated fruits and vegetables, or by soiled fingers. Playing on the ground is a common source of infection for children.

Laboratory diagnosis: discharge of the adult worm with the feces is common. In nearly all cases, diagnosis can be made by detection of the eggs.

5. Enterobius vermicularis

This, the pinworm, is worldwide in distribution. It matures in the small intestine and cecum and in the adult stage inhabits the colon and rectum, especially of young children. Infection results from ingestion of embryonated eggs. Gravid females usually migrate through the anus and deposit eggs in perianal folds, causing pruritis ani and vulvi. Adult worms are not infrequently found in feces, particularly after copious enema. The ova are rarely found in the feces. They are best found by scraping the skin at margin of anus with a dull knife, or with NIH swab.



The N I H Anal Swab

6. Trichuris trichiura (whipworm)

This parasite is 3.5 to 5 cm. long. Its anterior portion is slender and threadlike, the posterior portion thicker. It is one of the most common intestinal parasites in this country, and is of world wide distribution. It lives in the large intestine with its slender extremity embedded in the mucous membrane. As a rule, these worms do not cause any symptoms, however, the damage they do to the intestinal mucous membrane may favor bacterial invasion. Infection results from ingestion of embryonated ova.

The worms are rarely found in the feces. The ova are comparatively small, brown, ovoid in shape, and have a button-like projection at each end.

7. Filaria

Wuchereria bancrofti

This is the most important member of the filaria group. It is distributed throughout the world in temperate and tropical regions. Many species of Aedes, Anopheles and Culex mosquitoes serve as intermediate hosts. Man acquires the infection during the bit of an infected mosquito. The adults normally live in the lymphatics in the region of lymph nodes and the female deposits microfilariae which are carried to the blood stream. Lymphangitis may be produced with an associated high fever, enlargement of lymphatic glands and inflammatory swelling of the parts affected. Repeated attacks may result in permanent swelling (elephantiasis).

Diagnosis is based upon demonstration of the microfilariae in blood films. Blood for examination should be taken at night since in most cases the parasites are found in the peripheral blood only at this time.

Acanthocheilonema perstans

Infection with this parasite occurs during the bite of an infected midge (Culicoides). The adults inhabit chiefly the mesentery and retroperitoneal connective tissue. Apparently the infection is symptomless. Occurs in tropical Africa and South America. Diagnosis depends upon recovery of microfilariae in the blood stream.

Loa loa

Human infection is acquired through the bite of infected Chrysops flies. The adult worms normally live in the subcutaneous tissues through which they migrate back and forth, causing a temporary inflammation (fugitive swelling). This parasite is found mainly in Central Africa. Diagnosis can be made by recovery of microfilariae from peripheral blood during the day; by removal of adult worms from their tunnels; or by the filaria-group serological test.

Onchocerca volvulus (the blinding filaria)

The parasite is transmitted during the bloodmeal of infected black flies (Simulium). The adults inhabit the subcutaneous lymphatics and are usually enclosed in fibrocystic nodules. Several months are required for development of nodules after infection. Larvae deposited in the nodules may spread in the skin for a considerable distance, and in cases over 5 years duration, may penetrate tissues of the eye, eventually causing blindness. O. volvulus is common in Central Africa, Western Guatemala and Southern Mexico. Diagnosis may be made by demonstrating the presence of adults or microfilariae in excised nodules.

Dracunculus medinensis (Guinea worm)

Distributed in parts of Africa, India, Arabia, West Indies, Brazil and Guiana. Infection occurs through swallowing water containing infected crustaceans (cyclops). Larvae liberated in the stomach make their way to connective tissue and when mature migrate to a position just under the skin, frequently near the ankle joint. A blister forms on the skin just over the head of the worm. When the blister breaks and upon contact with fresh water, larvae are passed through this opening into the water.

The adult males vary in length from 12 to 30 mm., while the females are much larger and may reach a length of 1 meter. Diagnosis is based upon finding the worm under the skin.

8. Trichinella spiralis

Infection with this parasite is known as Trichinosis. It occurs mainly in the United States and Europe. Infection is acquired by eating raw or improperly cooked pork containing encysted larvae. Ingested females deposit larvae in the lymphatics of the small intestine, whence they are carried to all parts of the body to become encysted in striated muscle. The cysts are oval and about 0.45 mm. by 0.25 mm. in size. The encysted larvae may live 10 to 20 years, but eventually die and the cyst becomes calcified.

This parasite affects chiefly rats and hogs, although other animals are susceptible. Hogs become infected by eating rats, and in turn, rats acquire the infection through eating scraps of pork around abattoirs.

The symptoms produced depend upon the number of parasites ingested and may be very mild or very severe, and end fatally.

During the second and third week after infection in severe cases, there is high fever and prostation. Larvae may then be found in the circulating blood. From the 10th day on larvae begin to migrate into the muscle, giving rise to severe muscle pain, stiffness and disability.

Diagnosis must usually depend upon finding the encysted larvae in bits of excised muscle (deltoid or pectoral), or upon intradermal test.

Laboratory procedures for the diagnosis of Helminth infection are outlined in Section III.

SECTION III

EXAMINATION OF FECES AND LABORATORY DIAGNOSTIC PROCEDURES

EXAMINATION OF FECES

The normal stool consists chiefly of water, undigested and indestible remnants of food and enormous numbers of harmless bacteria. The quantity of feces varies with the amount and types of foods eaten, but normally averages about 200 Gms. a day. One or two stools per day may be considered normal, but one stool every three or four days may not be incompatible with health.

Macroscopic Examination

1. Odor - may be reported as normal, slight, sour, putrid, very foul, etc. The odor is influenced by diet and disease. It is stronger with meat than with a vegetable diet, and may be very slight when milk is the only food.

2. Color

    a. Light or dark brown is the normal color and is due to bilirubin.

    b. Yellow (may be due to milk diet, rhubarb, senna, santonin, unchanged bilirubin).

    c. Green (leafy vegetables, calomel, biliverdin).

    d. Clay (deficiency of bile).

    e. Acholic (undigested fat, jaundice).

    f. Dark reddish brown (excess of cocoa or chocolate).

    g. Black (iron, bismuth, charcoal, blood).

    h. Red (undigested blood, beets).

3. Consistency - fluid, semi-solid, formed, hard. With diarrhea or after cathartics, the stools are mushy or liquid. Constipation results in hard stools. Ribbon-like stools may result from some obstruction in the rectum.

4. Mucus - report as strings, balls, casts or ribbons, and approximate amount. Excessive quantities of mucus signify irritation or inflammation.

5. Blood - localized, diffuse, color, amount.

6. Concretions - should be searched for while washing feces through a sieve with water. Gallstones are usually faceted but may be definitely identified by testing for cholesterin and bile pigments.

7. Parasites - Some parasites may be detected by washing feces through a sieve and examining suspicious objects in a flat-bottomed dish over a dark background. (See also Section on Parasit.Lab. Methods).

Chemical Examination

1. Reaction - acid, alkaline, neutral.Test with litmus paper.

2. Occult blood - chemical tests for microscopic amounts of blood depend upon the reaction of iron in the liberated hemoglobin with the reagent employed. To avoid false positives, the patient should have been on a meat free diet for 72 hours prior to testing.

a. Benzidine Test

This test is most reliable when run on ether extract of feces. (See D'Riva's Concentration Method, Section III).

(1) Dissolve knife-tip of benzidine in 2 cc. of glacial acetic acid and add 1 cc. of hydrogen peroxide.

(2) Make a smear of feces on a slide and pour above reagent over it. The appearance of a blue color in a few seconds indicates a positive test.

b. Orthotoluidine Test

Reagent - 4% orthotoluidine in glacial acetic acid.

(1) To 1 cc.of watery feces, add 1 cc. of reagent.

(2) Add 1 cc. of hydrogen peroxide (3%). A positive test is indicated by the appearance of a bluish-green color.

3. Bile - since bilirubin is produced from hemoglobin, an estimation of urobilin in the feces gives some information as to the rate of blood destruction. In obstructive jaundice, urobilin is reduced in amount or absent from the feces.

a. Schmidt's Qualitative Test - emulsify a little feces in a saturated aqueous solution of mercuric chloride. Observe after 6 to 24 hours. Urobilin, the normal pigment, gives a salmon pink color; bilirubin, green. (This test takes place at once if the emulsion is boiled).

b. Gmelin's Test - make a thin smear of feces on filter paper and touch with a drop of yellow nitric acid. The appearance of a rainbow of colors with green on the outside indicates a positive test.

Microscopic Examination

To get a uniform mixture and representative sample for examination, it is best to rub up a portion of feces about the size of a walnut, in water. This may then be used for the preparations listed below.

1. Place a drop of feces suspension on a slide and apply a cover glass. This preparation should be just thick enough that newsprint can still be read through it. Examine with both low and high power, covering the slide systematically. The beginning student may first use this preparation for studying the elements found in normal feces, such as vegetable cells, plant hairs, etc. (See diagram in text). Other structures that may be observed in this preparation are listed below.

a. Degree of digestion of muscle fibers. If striations are visible, digestion is imperfect.

b. Red blood corpuscles are rarely seen unless their source is the rectum, colon or anus. Bleeding from the small intestine or higher, must usually be detected by a chemical test. Red cells may be present in clumps in amebic dysentery.

c. Pus cells are present in ulcerative conditions of intestine. They may be seen more clearly by adding a drop of 30% acetic acid to the slide.

d. Epithelial cells in small numbers are practically always present, but may be so degenerated as to be unrecognizable. Excessive number indicates inflammation or irritation.

e. Crystals of various types may be found, but are not of much significance. Triple phosphates, calcium oxalate, fatty acids and Charcot-Leyden crystals may be seen.

f. Ova and larvae of parasites may be detected in this preparation. (See also parasitological methods).

2. Mix a drop of feces emulsion on a slide with a drop of Lugol's iodine solution, apply cover glass and examine for protozoan cysts and starch grains. Undigested starch granules turn blue, partly digested ones appear reddish.

3. Mix a drop of the emulsion with 1 or 2 drops of Sudan III and examine for fats. Flakes or droplets of neutral fats stain orange red; normally no appreciable amount is present. Fatty acids appear as flakes or needles and stain very faintly. Soaps appear as yellowish flakes or coarse crystals.

4. Examination for parasites and ova should consist first of direct smear with and without Lugol's solution, and if unproductive, this should be followed in order by flotation preparation and a concentration method. (See parasitological methods)

Flotation Solutions

a.	Glucose	500 Gms.
	Water	320 cc.
b.	Sodium Chloride (sat. sol.)	
c.	Zinc Sulfate ($ZnSO_4$)	
	Specific Gravity	1.180
d.	Glycerin and saline, equal parts	

I. LABORATORY METHODS FOR THE INTESTINAL PROTOZOA

A. Collection of Specimens

Successful demonstration of the intestinal protozoa depends to a great extent upon the care with which the sample of feces is collected. If the trophozoite forms are to be identified, it is essential that the examination be made as soon as possible after a fluid specimen is passed, preferably within thirty minutes. It is well to have the specimen collected in the laboratory where the diagnosis is to be made. A clean, dry, covered receptacle should be used. Admixture with urine or antiseptics should be avoided. If a fluid specimen must be transported from ward to laboratory some means of keeping it at body temperature should be supplied. A container has been devised which consists of a fluted cylindrical base in which warm water is placed, and an enamelled bowl of slightly smaller diameter which fits in double-boiler fashion within the cylinder. A projecting edge holds the bowl at the top of the cylinder.

If the patient is not having an active diarrhea, a liquid stool specimen is obtained by administering a saline laxative. A specimen obtained by an oil cathartic is to be avoided. It is well to wait at least 72 hours after such a cathartic has taken effect before collecting a sample for examination. Microscopic droplets of oil are often confused with cysts. Specimens collected by an enema are also undesirable.

Formed stools for examination for cysts may be shipped to the laboratory if no facilities are available locally. Examination should be made within two days after the specimen is passed.

B. Preparation of the Specimen for Microscopic Examination

1. Direct Examination

Direct examinations for intestinal protozoa are made by emulsifying the feces with a drop of physiological saline on a glass slide. The mixture is smeared across the slide to the width of two cover glasses. A cover glass is placed immediately over one half of the smear. To the other half a drop of iodine stain is added, and a cover slip applied. In this manner the material may be examined, both stained and unstained, on the same slide.

With Lugol's and D'Antoni's iodine stains* the cytoplasm of the protozoan cysts appears lemon or greenish-yellow, while the cyst wall, nuclear membrane, and karyosomes are unstained and have a greenish-refractile appearance. Chromatoidal bodies, if present, appear as unstained refractile bars within the cytoplasm while glycogen vacuoles stain a yellowish-brown.

*See section on formulae for composition of all stains and reagents mentioned in this chapter.

With Kofoid's eosin-iodine stain, best results are obtained by placing a small drop of stain adjacent to the fecal suspension and placing a cover glass over both drops. A stained area and an unstained area result. In the clear area living organisms and unstained cysts appear. In the stained area the bacteria, fecal particles, and yeasts (except the larger forms) stain at once. Protozoan cysts stand out clearly as bright spherules which soon become tinged with the iodine to varying shades of yellow, while their glycogen inclusions, when present, turn light to dark brown according to their mass. As the stain penetrates, the nuclei become more clearly defined. This method provides a clear picture of the smear because of its differential staining characteristics.

If the first slide examined is negative, at least three other samples should be selected from different parts of the stool. Those portions containing blood or mucus are most likely to reveal parasitic protozoa.

In a fresh warm stool the parasites appear actively motile. Such motility may be maintained during the examination by the use of a warm stage apparatus. Examinations, especially for the vegetative forms of the amebas, may be aided by the use of a drop of vital stain which the trophozoites readily absorb without interfering with their motility.

Most infections of any importance are sufficiently heavy to be demonstrated in direct preparation. To discover the cysts in light infections, it is desirable to use a concentration method.

2. Concentration Methods

a. Simple Sedimentation

Where laboratory facilities are limited, the simple sedimentation method may be used. A portion of the stool about the size of a pecan is emulsified in a test tube in about ten parts of lukewarm water. If no centrifuge is available, the tube is allowed to stand until the sediment settles out. The supernatant is decanted and 2 or 3 cc. of water are added to the sediment which is then broken up. The tube is nearly filled with water, the contents mixed well and the process of centrifuging or settling is repeated. These steps should be repeated until the supernatant is clear.

b. Zinc Sulfate Flotation Method

A more efficient method which may be used with limited equipment is the flotation method. A solution of high specific gravity is used, and the cysts of the protozoa are floated to the surface of the liquid. Most commonly used is a 33% aqueous solution of zinc sulfate. The specific gravity of this solution is about 1.18. The technic is as follows:

(1) Suspend a portion of the stool, about the size of a pecan, in 10 quarts of lukewarm water.

(2) Strain through wet cheesecloth in a small funnel into a Wasserman tube.

(3) Centrifuge at 2500 rpm for 45 to 60 seconds. Pour off the supernatant, add 2 to 3 cc. water, break up the sediment, and add more water.

(4) Repeat step number 3 until the supernatant is clear.

(5) Pour off the last supernatant, add 3 to 4 cc. of the zinc sulfate solution, break up the sediment, fill to within one-half inch of the top of the tube, and centrifuge at 2500 rpm for 45 to 60 seconds.

(6) Remove the diagnostic material at the surface of the tube with a wire loop. Add a drop of iodine stain and make a cover-slip preparation.

In the absence of a centrifuge the fecal material may be emulsified in plain water and allowed to settle out repeatedly until the supernatant is clear. Then the remaining sediment may be mixed with a zinc sulfate solution in a shallow dish. After an hour or more, during which time the material has been mixed frequently, the diagnostic surface film may be removed by the application of a cover glass to the liquid.

Other solutions may be substituted for the zinc sulfate as long as the specific gravity remains at about 1.18 to 1.2. A 24% solution of sodium chloride or a 40% solution of ordinary cane sugar may be used.

c. DeRiva's Method

Then the materials and equipment are available, the most desirable concentration method is that described by DeRiva:

(1) Emulsify approximately 1 gram of feces in 5 cc. of 5% acetic acid.

(2) Strain through wet cheesecloth into a graduated centrifuge tube.

(3) Add an equal volume of ether and shake vigorously for 30 seconds to 1 minute.

(4) Centrifuge at 2500 rpm for 5 minutes.

(5) Remove the sediment from the bottom of the tube with a capillary pipette and make a cover-slip preparation.

Four distinct layers result: First, the ether layer, which may be used for an occult blood test; second, the detritus plug, composed of layers of bile, soaps, and protein material; third, the acetic acid solution; and fourth, the sediment which will contain the cysts or ova.

In the course of a routine stool examination, one should make at least four direct preparations to be examined with and without iodine stain, and in addition, one of the concentration methods should be performed and the sediment viewed direct and with iodine stain.

C. Special Staining Methods

For routine purposes the temporary iodine stains are usually sufficient. For some diagnostic work and for permanent mounts, it is desirable to use a stain which clearly defines the cell structures. The standard stains for such work contain the dye hematoxylin.

The feces to be stained may be smeared, or stained in bulk. To prepare a smear for demonstration of trophozoites, a bit of the feces is spread on a cover glass. The specimen is usually sufficiently fluid so that the preparation of a saline suspension is unnecessary. The cover-slip is then immediately dropped, smear down, in a watch glass of Carnoy's or Schaudinn's fixative. The fixative is allowed to act for 20 to 30 minutes. If the smear has been made properly on a perfectly clean cover glass, there will be little loss of the trophozoites during fixation.

Solid or semi-solid specimens may be emulsified in saline, smeared on cover glasses and treated as above. If difficulty is experienced in making the trophozoite material adhere to the cover glass, it may be smeared on a glass slide which has been rubbed with egg albumin or horse serum. A cover glass is then applied and the smear placed in an empty Coplin jar. The fixative is slowly poured into the jar. The smear is allowed to remain in the fixative from 20 to 30 minutes. The slide is then removed and the cover glass is taken off.

After fixation the smears are stained according to one of the methods given below:

1. Technic for Heidenhain's Iron-Hematoxylin Stain

a. Remove smears from fixative and rinse.*

b. Mordant in 5% aqueous iron alum for at least 45 minutes.

c. Rinse in water.

d. Stain in Heidenhain's Iron Hematoxylin for at least 45 minutes.

e. Rinse in water.

f. Differentiate in 2 to 5% iron alum, controlling the degree of definition with the microscope.

g. Wash in running water for 2 to 5 minutes.

h. Dehydrate in ascending grades of alcohol. (50%, 70%, 95%, absolute).

i. Clear in xylol.

j. Mount in Canada balsam.

Since this stain must be used regressively, i.e., the material is grossly overstained and then differentiated, its success depends entirely on accurate differentiation. A common error is to fail to carry the process of differentiation far enough. A 2% solution of iron alum extracts the stain more slowly, and is most convenient for the beginner.

If the smears are accidentally over-differentiated, they may be replaced in the hematoxylin bath until they are jet black, then redifferentiated.

Iron hematoxylin is a permanent stain. It never fades, provided the iron alum is properly rinsed out of the smear after differentiation. A large number of counterstains may be used with the hematoxylin stain.

*Use 90% alcohol in several changes after Carnoy's. If Schaudinn's or any other fixative containing mercuric chloride is used, it is necessary to remove the deposit of mercuric chloride before staining. This is done in the following manner:

(1) Immerse in 70% alcohol for 10 minutes.

(2) Immerse in 70% alcohol to which enough iodine stain has been added to impart a mahogany color for 10 minutes.

(3) Immerse in 70% alcohol for 10 minutes.

2. Technic for Harris' Hematoxylin Stain

a. Remove fixative and rinse in the proper medium.

b. Stain in Harris' Hematoxylin for at least 45 minutes.

c. Wash in tap water.

d. Destain in acid alcohol (1% HCl in 70% alcohol) Check the degree of differentiation with the microscope.

e. When the desired definition has been reached, transfer to dilute ammonia water (5 drops of ammonium hydroxide in 50 cc. of water). Allow to neutralize until the smear is completely blue.

f. Dehydrate in ascending grades of alcohol.

g. Clear in xylol and mount in balsam.

3. A Method for a Rapid Hematoxylin Stain

This method is especially useful in diagnostic work, where permanence is not required.

a. Make a smear of the feces.

b. Fix while wet, and rinse according to the fixative.

c. Mordant for 2 to 3 minutes in 5% iron alum at 56 degrees Centigrade.

d. Rinse in water.

e. Stain in Hematoxylin for 1 to 2 minutes at 56°C.

f. Rinse in water and allow to stand until blue-black.

g. Dehydrate in 95% alcohol 30 seconds to 1 minute, followed by absolute alcohol or acetone for 1 minute.

h. Clear in xylol and mount in balsam.

The Hematoxylin used in this staining method is made by adding 0.4 cc. of 10% alcoholic solution of Hematoxylin and 0.8 cc. of glacial acetic acid to 40 cc. of distilled water.

Many workers prefer to stain protozoan material in bulk. This method overcomes certain difficulties usually encountered when smears are used: First, keeping the organisms on the slide or cover glass during fixation; second, keeping the organisms free from distortion and clear from debris so that the internal structures are not obscured; third, carrying out proper differentiation of internal structures of the organisms on the slide at the time of differentiation; fourth, having a sufficient number of well stained organisms on the slide after staining so that the diagnosis will not have to be made on a few more or less atypical organisms. The bulk staining method also affords a method of concentration while staining. Very few organisms are lost, and distortion is held to a minimum.

4. Bulk Staining with Heidenhain's Iron-Hematoxylin

a. Fix with Carnoy's in 50 cc. centrifuge tubes for at least 30 minutes.

b. Decant the supernatant. (Centrifuging between steps is necessary only when washing, or after differentiation).

c. Wash with 90% alcohol, several changes.

d. Wash with 70% alcohol.

e. Wash with 50% alcohol.

f. Stain with Heidenhain's Iron-Hematoxylin:

(1) Mordant in 5% iron alum for 45 minutes or more.

(2) Rinse in water.

(3) Stain in Hematoxylin for 45 minutes or more.

(4) Rinse in water.

(5) Differentiate in 5% iron alum.

(6) Wash with several changes of water.

g. Dehydrate in ascending grades of alcohol.

h. Clear in xylol.

i. Add a balsam-xylol mixture.

The degree of differentiation is controlled by making a cover glass preparation of the material at intervals while it is destaining, and examining with the microscope.

Harris' Hematoxylin may be used, following the same general principles used with Heidenhain's:

a. Fix and rinse.

b. Stain in Harris' Hematoxylin for 45 minutes or more.

c. Wash in tap water.

d. Destain in 1% HCl in 70% alcohol, checking continually with the microscope to control the degree of differentiation.

e. Neutralize with dilute ammonium hydroxide.

f. Dehydrate in ascending grades of alcohol.

g. Clear in xylol.

h. Add a balsam-xylol mixture.

D. Culture Methods

1. Boeck-Drbohlav Medium. (Dobell and Laidlaw's Modification)

Four eggs are carefully washed, brushed with alcohol, and broken into a sterile flask containing glass beads. 50 cc. of sterile Ringer's solution are added and the mixture thoroughly shaken until a homogenous suspension is secured. The mixture is tubed in 4 cc. lots, or enough to produce a slant of about 1 to 1 1/2 inches when coagulated by heat. The tubes are then slanted and placed in an inspissator and kept at 70°C., until the slants are solidified. The tubes are then autoclaved at 15 pounds pressure for 20 minutes. After autoclaving, the slants are covered to a depth of about 1 cm. with a mixture of 8 parts of sterile Ringer's Solution and 1 part of sterile inactivated human blood serum. To insure sterility, the mixture of Ringer's Solution and blood serum should be passed through a Berkfeld filter and incubated at 37°C. for at least 24 hours before it is used. A bit of sterile rice powder should be added to each tube when culturing the amoebas.

The tubes are inoculated with an applicator or wire loop, selecting a pea-sized portion of the specimen containing fresh mucus or mucus and blood; a bit of concentrate containing cysts may also be used. The culture is incubated at 37°C. and examined at 24 hours and at 48 hours. Flagellates will be found throughout the fluid portion of the media. Amoebas will be found at the base of the fluid portion.

A sterile 1 cc. pipette is used to withdraw 0.1 cc. of the fluid for examination. Routine cultures are transferred every 48 hours.

If a new culture is not positive after 48 hours, all but 0.5 cc. of the fluid portion is removed; the slant is washed with the remaining fluid, and transferred to a new tube of media. The resulting culture should be examined at 24 and 48 hours before being pronounced negative.

The amoebas are quite sensitive to the presence of certain species of bacteria, especially Pseudomonas aeruginosa and Proteus vulgaris, and die readily in their presence. Other organisms, particularly Escherechia coli are beneficial in the culture. It is essential that aseptic technic be followed in preparing the culture media.

II. LABORATORY METHODS FOR BLOOD AND TISSUE PROTOZOA

A. Methods for Examination for Malaria

1. Fresh, Unstained Blood

With practice, the malaria parasites may be detected and diagnosed from the study of a fresh specimen. A cover glass is touched to a drop of blood, and placed on a slide. The preparation is then examined with the 1.8 mm. objective. Movement of the pigment granules within the parasite may be detected without the use of a warm stage in the tropics. Such examinations require the use of subdued light. If the preparation is to be studied for any length of time, the cover glass should be ringed with vaseline.

2. Thin Smear Method

Blood smears are prepared as for leukocyte counts, except that they are spread more thinly to provide an undistorted picture of the erythrocytes. The smears are stained as for differential leucocyte counts. The nuclei of the leukocytes must be heavily stained if the parasites are to be well defined.

3. Thick Smear Method

a. Slides for this purpose must absolutely be clean and free from grease.

b. Four small drops of blood are placed on a slide so that each drop marks the corner of a square which could be covered by a dime. The drops are coalesced with an applicator or a toothpick. Some workers use a single large drop, which is then spread over the same area as that obtained in the four-drop method. If too much blood is used and the resulting smear is too thick, the blood film will crack and peel from the slide. If too little blood is employed, the parasites will be too sparse. The exact amount of blood to be used is easily determined after short experience.

c. The smear is allowed to dry long enough to make it adhere, but not long enough to prevent clear staining of the parasites. 1 1/2 hours in an incubator at 37°C. is usually a sufficient time. The smears should be protected from dust and flies while they are drying.

d. When film is quite dry it is ready for staining. Proceed as follows:

(1) Dehemoglobinize by flooding slide with a solution of 0.1% magnesium sulfate in distilled water. Allow to stand 5 to 10 minutes.

Note: an alternate solution that can be used for dehemoglobinizing and fixing where more permanent mounts are desired, is the following:

Formalin	5 cc
Acetic acid	1 cc
Distilled water qs ad	100 cc

Allow slide to remain in this solution 10 minutes (thick film only).

(2) Flush by pouring tapwater carefully over the slide from one end, out of a measuring glass. A white streak consisting of leukocytes, platelets and the stroma of erythrocytes should be left.

(3) Drain tapwater from slide and allow to dry while resting obliquely against some object.

(4) When slide is quite dry, it may be laid horizontally and flooded with a mixture of 2 drops Giemsa's Stain in 2 cc. of water. (Water used here should have a pH of about 7. If this is not available, use a 1/1000 solution of magnesium sulfate in distilled water).

(5) Allow stain to remain on slide for 20 to 40 minutes or more, as indicated by previous staining experience.

(6) Flush by pouring water carefully over the slide from one end, taking care not to allow metallic looking film floating on the stain to come in contact with bloodfilm.

(7) Dry and examine. (Slide should never be dried with artificial heat or by placing in sun).

The slide should not be left in the stain for less than 20 minutes. If after this period, the slide is too strongly colored, this may be taken as an indication for further dilution of the stain on the next occasion.

If the thick drop is properly stained, the microscope reveals a colorless or slightly yellowish background, not blue. Platelets will appear red-violet; leukocytes will be strongly colored; stroma of erythrocytes should be visible as a blue network.

Malaria parasites will have a red nucleus and dark blue cytoplasm. Where present, Schuffner's or Maurer's dots should be visible. Films that are stained too lightly do not show these dots and the cytoplasm will be a pale blue. When staining is too strong the parasites are shriveled and may be covered with red streaks (fibrin) and blue coloring matter. Films that are old or have been subjected to the action of light will be more or less fixed and will be unusuable.

e. The thick smear may be combined with the thin smear on the same slide. In this case, the thin smear is immersed in methyl alcohol for fixation, but is not dehemoglobinized, and both smears are stained with Giemsa's. The thick smear may be used to determine the presence of the parasites, and their morphology may be studied in the thin smear.

4. Barber and Komp Method for Handling Large Numbers of Thick Films in a Malaria Survey

"In handling large numbers of thick smears, it is convenient to carry out the technique in groups of 25 slides. With this in mind, the thick film is placed about 1 inch from one end of the slide and the other is used for labeling. The slides are assembled in groups, a cardboard one sixteenth to one eighth of an inch thick and 1 1/2 inches long, is inserted between the slides at the labeled ends and the whole fastened together by means of a stout rubber band. The entire block may now be stained and dried as a single unit.

The combined thick and thin smears for staining are prepared by making a thick smear on one end of the slide and a thin smear starting 1/2 inch from the thick smear, and then streaking it toward the opposite end of the slide. Draw a line with a wax pencil between the two smears and they are now ready for staining. Proceed as for a thick smear but be careful to immerse only the thick smear in the acidulated formaldehyde solution. If the thin smear comes in contact with this solution, the red cells will be dissolved out and the smear will be useless.

Failure to stain by Wright's method is usually due to insufficient lapse of time after diluting the stain with distilled water, or to contamination of the stain, or other reagents, or material, with acid. The precipitation of granules of stain on the blood film is either due to improper drying of blood films before starting the stain,

introduction of water into the stock stain, or too much evaporation of the alcoholic stain before dilution. Red cells stained blue, except for the occasional cells showing polychromatophilia, are either overstained (too much time allowed after diluting the stain), or have been insufficiently washed during the last stage of the staining process."

5. Concentration by the Method of Bass and Johns

The equipment required, length, and difficulty of this procedure do not make it desirable for routine work. It is valuable as a check on therapy, and will uncover cases not easily demonstrated by the preceding methods. It utilizes the principle that the parasitized erythrocytes are lighter than the normal cells. Best results are obtained with estivo-autumnal crescents and with the adult stages of the other species.

a. Draw venous blood and place it in a tube containing the proper amount of dry potassium oxalate. (2 mg. per cc. of blood)

b. Centrifuge at 2500 rpm for 5 minutes.

c. Withdraw most of the plasma with a capillary pipette, and place it in a small tube.

d. Carefully skim off the leukocyte layer and the upper layer of erythrocytes and place them in a tube 12 cm. by 1 cm., inside measurements. Add an equal volume of plasma.

e. Mix and centrifuge as before.

f. Draw off the leukocyte layer and the upper erythrocyte layer into a long capillary pipette of about 3 to 4 mm. bore. Mix by forcing in and out on a slide. Finally draw the well-mixed upper cell layer into the pipette, and seal the tip in a flame. Nick with a file and break above the blood column.

g. Place the tube thus formed in a centrifuge tube, pack with cotton, and centrifuge as before.

h. Nick with a file and break 1 to 2 mm. below the bottom of the leukocyte layer.

i. From the upper section of the tube, with a capillary pipette small enough to enter the bore of the capillary tube, remove the small amount of red cells together with a little plasma and the leukocyte layer.

j. Mix well, smear and stain with Wright's or Giemsa's.

B. Methods of Examination for Trypanosomes

1. Fresh Preparations: the organisms are best demonstrated by the use of darkfield illumination. The living parasites actively displace the surrounding red cells.

2. Thin Films: the films are prepared and stained in the standard manner.

3. Thick Films: it is possible to employ thick films prepared as for malaria, but considerable distortion of the organisms occurs.

4. Concentration Method: blood may be concentrated by centrifuging 10 cc. of oxalated blood and making smears of the leukocyte layer. The smears are stained with Wright's or Giemsa's.

5. Lymph Node Aspiration: Smears may be made from lymph node fluid obtained by aspiration and stained with Wright's or Giemsa's. This method is used when examination of the peripheral blood is negative and often demonstrates the organisms in the early stages of the disease.

6. Spinal Fluid: spinal fluid is obtained by lumbar puncture and centrifuged for 15 minutes. Smears are made of the sediment and stained with Wright's or Giemsa's. This method is useful in the later phases of sleeping sickness when other methods are negative, but should not be expected to demonstrate organisms before the encephalitic stage is well developed.

7. Animal Inoculation: white rats are injected intraperitoneally with 1 cc. of blood or tissue juice. Daily blood examinations are made. In positive cases, the trypanosomes will appear in the blood of the animal between the third and fourteenth days and remain quite constantly.

C. Diagnostic Methods for the Leishmanias

1. Spleen and Liver Punctures: material should be obtained only by an experienced medical officer. It is spread on a slide in a thin layer and stained with Wright's or Giemsa's, and examined with the 1.8 mm. objective. In positive cases, Leishman-Donovan bodies will be found within the reticulo-endothelial cells.

2. Peripheral Blood: Leishman-Donovan bodies are found in the blood in only about 20% of cases. They will be found, if present, within the monocytes or occasionally within the polymorphonuclear leukocytes.

3. From Ulcerations: in cases of Oriental Sore, the Leishman-Donovan bodies may be demonstrated in endothelial cells obtained from scrapings from the ulcer margins.

4. Culture:

NNN Medium (Novy - MacNeal - Nicolle Medium)

Agar	14 gm.
Sodium chloride	6 gm.
Distilled Water	900 cc.

Mix and dissolve by means of heat. Tube in 6 cc. amounts. Autoclave 30 minutes at 20 lb. pressure. Remove and cool to 48°C. Add aseptically 2 cc. of sterile defibrinated rabbit's blood to each tube, mix well, and slant. The tubes should be cooled in the ice box to produce a maximum amount of water of condensation. They should be capped with rubber stoppers to prevent excess evaporation of this water of condensation. Incubate for 24 hours to test sterility. Inoculate into the water of condensation and incubate at 22 to 25°C. for 3 to 14 days. In cultures, the Leishmania will be found in the Leptomonas or flagellated stage.

III. EXAMINATIONS FOR HELMINTHS

The ova of the intestinal helminths may be recovered by direct examination of a saline emulsion of the stool, or by one of the methods of concentration discussed under the topic "Methods for Intestinal Protozoa". The life cycle of the parasitic helminths must be considered when selecting the proper specimen for examination. Thus, rust colored flecks in the sputum would be examined for the ova of Paragonimus westermanii, and urine for the ova of Schistosoma hematobium.

A. Examination for Filaria

Diagnosis of Filaria bancrofti, Loa loa, and Filaria perstans is established by examining the peripheral blood as follows:

1. Direct Method: a drop of blood obtained at the appropriate hour is covered with a cover glass and examined under low power for microfilaria. They may be easily detected by following the disturbance of the surrounding red cells.

2. Concentration Methods

a. 1 cc. of blood is added to 2 cc. of a 2% solution of acetic acid. It is mixed well, centrifuged, and the sediment spread on a slide. A cover slip is placed over the wet smear, and the preparation is examined under low power.

b. 20 drops of blood are added to 10 cc. of physiological saline plus a few drops of a 10% solution of saponin. After hemolysis, the mixture is centrifuged and the sediment examined for living microfilaria.

c. A solution of 5% formalin, 5 parts, and saturated alcoholic gentian violet, 2 parts is added to the blood. The mixture is then centrifuged, the supernatant discarded, and the residue covered with water. It is then recentrifuged, and the sediment examined for microfilaria.

B. Examination for Strongyloides stercoralis

In the identification of the rhabditiform larvae of Strongyloides it is a good policy to base the diagnosis on living forms, as certain vegetable spines and hairs are sometimes confused with the larvae. Usually the larvae may be found in a direct fecal film, but concentration methods give a much richer yield. In a solid stool specimen the larvae may be found by making a small depression in the fecal mass, filling it with water, and keeping it in a warm place for 12 to 24 hours. The larvae collect in the water. Some means of keeping the stool moist must be provided.

C. Examination for Enterobius vermicularis

The ova of Enterobius vermicularis are not normally recovered in abundance from the feces. Swabbing or mildly scraping the perianal regions yields a larger number of eggs. Adults may be captured following enemas, or females may be recovered during their normal nocturnal migration from the bowel.

The NIH swab is most commonly used in the recovery of the ova of Enterobius vermicularis. It is prepared as follows:

1. Fix a glass rod (4 mm.) in a rubber stopper in such a manner that it will reach almost to the bottom of a 15 x 85 mm. test tube when the stopper (No. 00, one-hole) is fitted in place. A short length of the rod should protrude from the top of the stopper.

2. Fasten a piece of cellophane 1 inch square over the tip of the glass rod with a rubber band made from rubber tubing of the appropriate diameter.

3. Use of the NIH swab:

Swabbing should be done in the morning before the patient has bathed or defecated. The dry cellophane-covered tip is stroked firmly with an outward motion over the perianal folds and across the anal opening. The cellophane is released by sliding the rubber band towards the stopper. The square is mounted in water on a glass slide and examination is made for the characteristic ova. Defects in the cellophane resembling the ova in outline should be recognized, as they are confusing and may lead to error.

D. Examination for Tapeworm Proglottids

When the segments of tapeworms are found in the stool, it is necessary to determine whether they are from the beef or pork tapeworms. This is done as follows:

1. Clean and relax the segments by shaking them in physiological saline.

2. Press the specimen between two glass slides.

3. Count the lateral branches of the uterus by holding the segment up to a strong light. The branches should be counted at their bases since there is some subdivision at the distal point.

IV. FORMULAE FOR REAGENTS USED IN PARASITOLOGICAL LABORATORY METHODS

A. Lugol's Iodine Stain

Iodine	1 gm.
Potassium Iodide	2 gm.
Distilled Water	100 cc.

B. Kofoid's Eosin-Iodine Stain

Eosin saturated in saline	2 parts
Iodine solution*	1 part
Physiological saline	2 parts

C. D'Antoni's Standardized Iodine Solution

A standardized solution of potassium iodide is prepared by the specific gravity method:

1. Place 100 gm. of Merck's or Baker's potassium iodide in a chemically clean 1000 cc. volumetric flask. Add distilled water to the mark.

2. Weigh a clean, dry 25 cc. volumetric flask to the 4th decimal place.

3. Fill the 25 cc. volumetric flask to the mark with the potassium iodide solution and weigh to the 4th decimal place.

*Physiological saline	100 cc.
Potassium iodide	5 gm.
Iodine crystals to saturation	

4. Subtract the weights in "2" and "3" to obtain the weight of the 25 cc. of potassium iodide solution. Theoretically this should be 26.925 gm. The actual weight will be slightly less than this, due to the deliquescence of the potassium iodide.

5. The difference in weight is divided by the theoretical weight, and the quotient expressed in terms of percentage. This quotient is subtracted from 10 (the percentage desired) to give the actual percentage of the solution.

6. The following proportion may then be set up:

$$\frac{100}{\text{Actual \% of soln.}} = \frac{x}{10\%}$$, where x = grams of potassium iodide.

7. 100 subtracted from the number of grams obtained from x gives the number of grams of potassium iodide to be added to the above solution to give a standardized 10% potassium iodide solution.

The staining solution is prepared by adding 1.5 grams of powdered iodine crystals to 100 cc. of a 1% potassium iodide solution which is obtained from the standardized 10% solution. The resulting solution is allowed to stand for 4 days and is then ready for use. It must be filtered before using, and should not be allowed to remain unstoppered, as volatilization of the iodine will occur. The stock solution keeps for long periods of time without deterioration.

D. Vital Stain

This stain is based on the 1% aqueous neutral red solution recommended by Stitt.

It has been found that when the neutral red is prepared in physiological saline in a concentration of 1%, and 6 drops of a saturated solution of Janus green in physiological saline are added to each 5 cc. of the neutral red solution, an efficient vital stain for protozoa is produced.

E. Heidenhain's Iron Hematoxylin Stain

1. The Alum Bath. (Used as mordant and differentiator).

Iron and ammonia alum $(NH_4)_2.Fe_2(SO_4)_4.24\ H_2O$)	5 gm.
Distilled water	100 cc.

2. The Hematoxylin Bath

Hematoxylin	.5 gm.
96% or absolute alcohol	10 cc.
Distilled water	90 cc.

A good brand of hematoxylin is essential. Dissolve it first in the alcohol; then add the water. The solution takes a few weeks to ripen. Best results are obtained by placing in a stoppered flask and exposing it daily to the sunlight. Frequent agitation hastens the ageing.

F. Harris' Hematoxylin

Hematoxylin	1 gm.
96% ethyl alcohol	10 cc.
Dissolve	
Alum (ammonium or potassium)	20 gm.
Distilled Water	200 cc.

Dissolve the alum in the water with the aid of heat and add the hematoxylin solution. Bring the mixture to a boil as rapidly as possible and add 0.5 gm. of yellow oxide of mercury. The solution at once becomes a dark purple. As soon as this occurs, remove the vessel from the flame and cool rapidly by plunging into a basin of ice water. As soon as the solution is cool it is ready for use. The addition to the solution of glacial acetic acid in a concentration of 4% brings out the nuclear components more clearly.

G. Schaudinn's Fixative

Mercuric chloride, conc. aq. soln.	2 parts
Absolute alcohol	1 part

H. Carnoy's Fixative

Absolute alcohol	6 parts
Chloroform	3 parts
Glacial acetic acid	1 part

I. Ringer's Solution

Sodium chloride	9.0 gm.
Calcium chloride	0.2 gm.
Potassium chloride	0.2 gm.
Distilled water	1000 cc.

SECTION IV

SOME ARTHROPOD VECTORS OF DISEASE

SOME ARTHROPOD VECTORS OF DISEASES OF MAN

VECTOR	ETIOLOGICAL AGENT	DISEASE	METHOD OF INFECTION
Cyclops	(Guinea worm) Dracunculus medinensis		Oral (water
	Diphyllobothrium latum		Oral (Thru fish as 2d intermediate host)
Crabs	Paragonimus westermanii		Oral
Body Louse (Pediculus humanus)	Borrelia recurrentis	Relapsing fever	Contamination of bite
	Rickettsia quintana	Trench fever	Contamination of bite
	Rickettsia prowazeki	Typhus fever	Contamination of bite
Rat Flea (Xenopsylla cheopsis)	Pasteurella pestis	Bubonic plague	Contamination of bite
	Rickettsia prowazeki	Typhus fever	Contamination of bite
Kissing Bug (Triatoma)	Trypanosoma cruzi	Chagas' disease	Contamination of bite
Mosquitoes Anopheles (70 species)	P.falciparum, P. malariae, P. vivax	Malaria	Bite
Anopheles (22 species)	Filaria bancrofti	Filariasis	Invasion of bite
Aedes (18 species)	Virus of yellow fever	Yellow fever	Bite
Aedes aegypti	Virus of Dengue	Dengue fever	Bite
Aedes (8 species)	Filaria bancrofti	Filariasis	Invasion of Bite
Culex (5 species)	Filaria bancrofti	Filariasis	Invasion
Sand Fly (Phlebotomus)	Virus of Pappataci fever	Pappataci fever	Bite
	Leishmania tropica	Oriental Sore	?
	Leishmania donovani	Kala Azar	?
	Leishmania braziliensis	Espundia	?
Tsetse Fly (Glossina)	Trypanosoma gambiense	African Sleeping Sickness	Bite
	Trypanosoma rhodesiense		Bite
Ticks (Dermacentor)	Pasteurella tularensis	Tularemia	Bite
	Rickettsia rickettsi	Rocky Mountain Spotted Fever	Bite
Mites (Trombicula)	Rickettsia japonica	Tsutsugamushi Fever	Bite

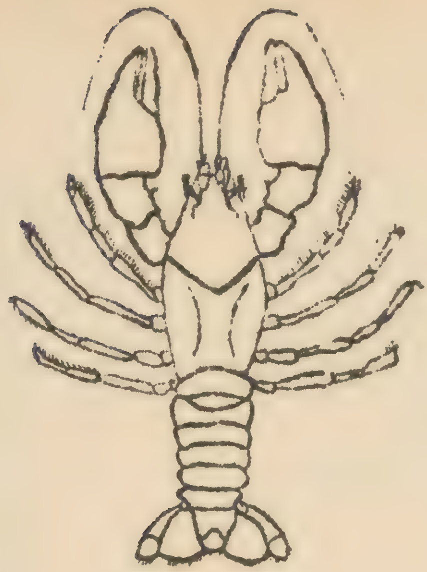
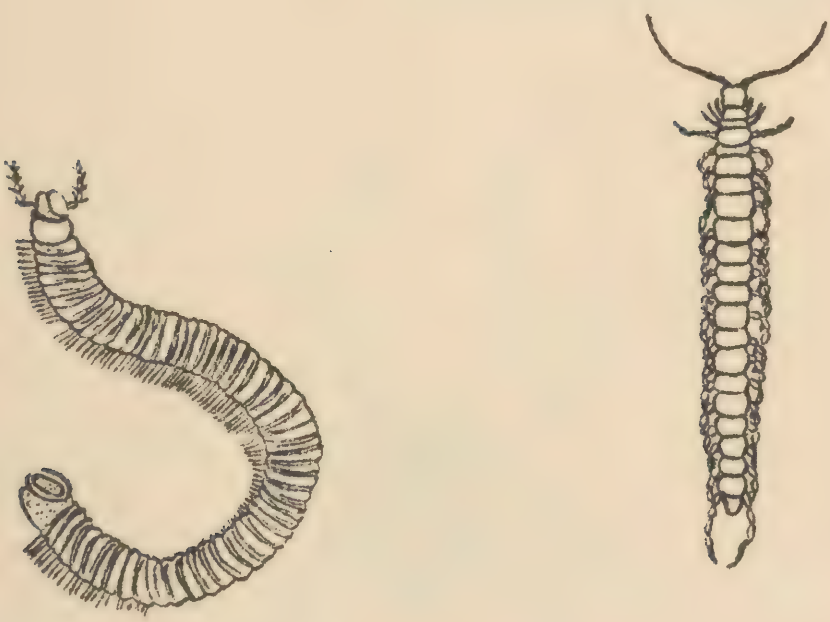
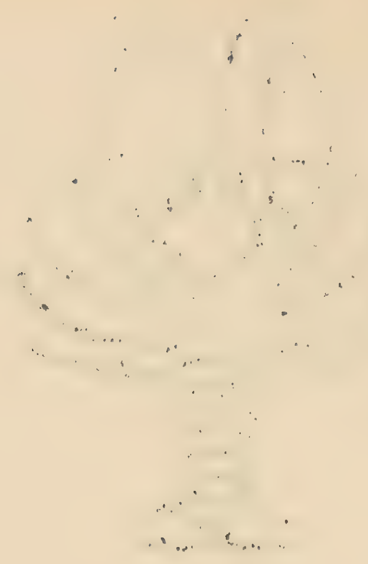


Fig. 1. Crayfish (a crustacean)



A

Fig. 2. Myriapods. A, Centipede; B, Millipede.



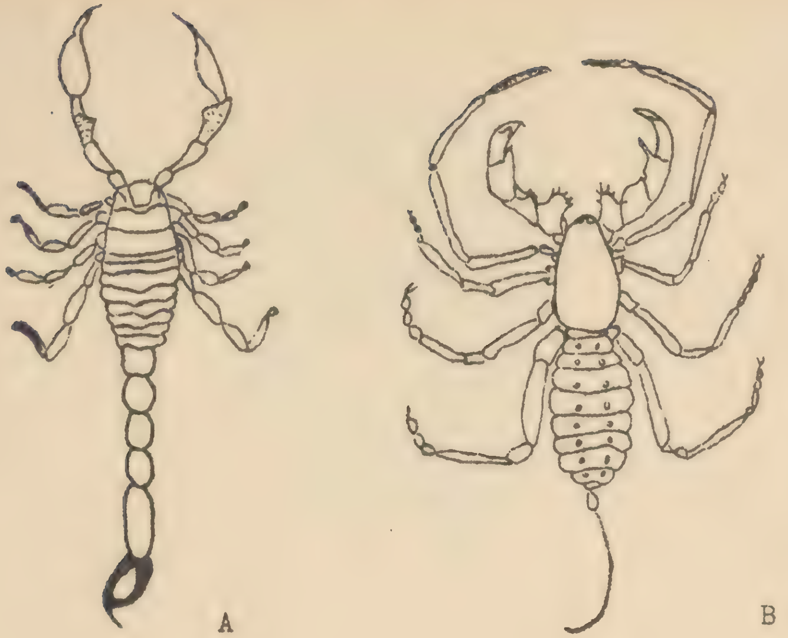


Fig. 3. Comparison of scorpion and whip-scorpion.

A, Scorpion; B, Whip-Scorpion.

Fig. 4. Black widow spider (an arachnid).

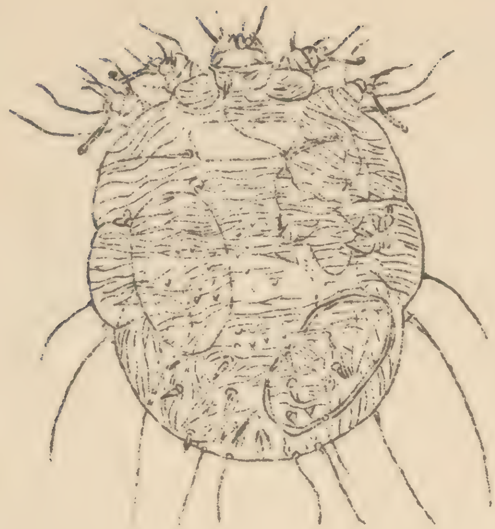


Fig 5 Itch-mite of man

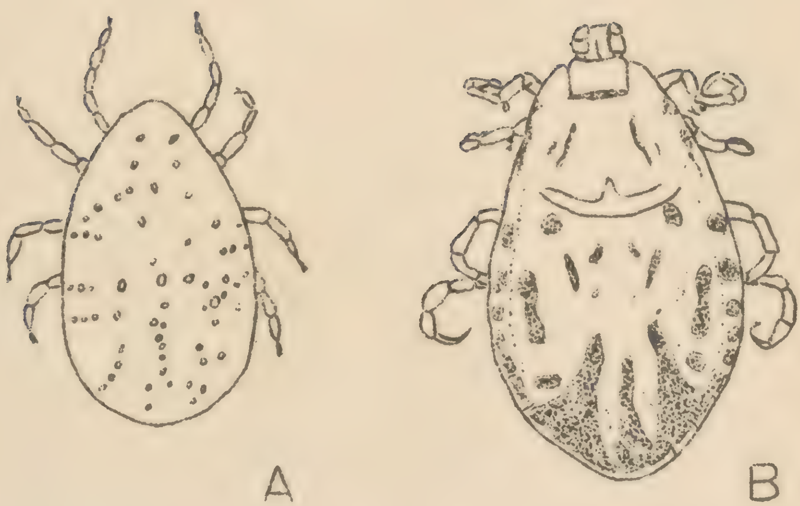
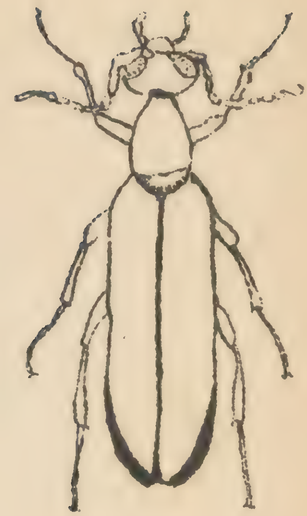


Fig. 6. Ticks A, Soft-bodied tick;
B, Hard-bodied tick

A

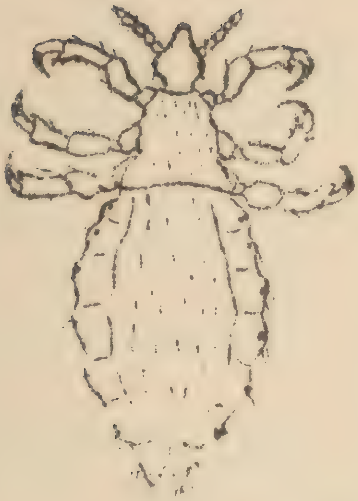


B

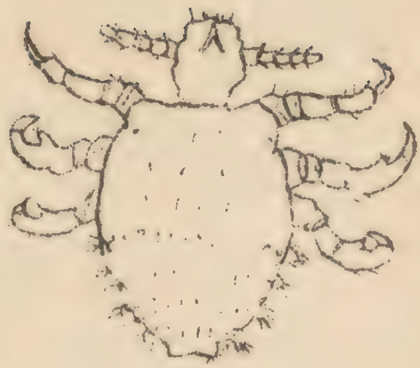
C

D

Fig.7. Insects. A, Cockroach (Orthoptera); B, Beetle (Coleoptera); C, Moth (Lepidoptera); D, Ant (Hymenoptera).



A



B

Fig.8. Lice (Anoplura). A, Body louse;
B, Crab louse.

B

Fig.9. Flies. A, Larva of house-fly;
B, Pupa of house-fly.

GEOGRAPHICAL DISTRIBUTION OF SOME ANOPHELINE VECTORS OF MALARIA

NORTH AMERICA	CENTRAL AND SOUTH AMERICA AND WEST INDIES	EUROPE	AFRICA	ASIA AND EAST INDIES	OCEANIA AND AUSTRALIA
A. cruciens	A. cruciens	A. hyrcanus	A. umbrosus	A. hyrcanus	A. annulipes
A. maculipennis	A. pseudopunctipennis	A. maculipennis	A. maculipennis	A. maculipennis	A. punctulatus
A. punctipennis	A. apicimacula	A. hispaniola	A. funestus	A. umbrosus	A. bancrofti
A. pseudopunctipennis	A. intermedius	A. superpictus	A. gambiae	A. barbirostris	A. subpictus
A. quadrimaculatus	A. pseudomaculipes		A. hispaniola	A. culicifacies	
	A. punctimacula		A. pharaensis	A. fluviatilis	
	A. albimanus		A. superpictus	A. maculatus	
	A. albitarsis			A. minimus	
	A. argyritarsis			A. philippinensis	
	A. darlingi			A. stephensi	
	A. tarsimaculatus			A. superpictus	
	A. gambiae (imported)				

DIFFERENTIATION OF MOSQUITOES

ANOPHELES	CULEX	AEDES
Adult Antennae	Adult of males are busy, those less branched	Adult of females
Palpi of female as long as proboscis	Palpi of female short	Palpi of female short
Palpi of male long and spatulate	Palpi of male are long	Palpi of male longer than proboscis
Wings spotted	Plain wings	Plain wings
Scutellum arculate	Scutellum trilobate	Scutellum trilobate
Rests at 50 to 80 degree angle with the surface	Rests in a plane horizontal to the surface	Rests in a plane horizontal to the surface
Body parts in a straight line	Head and proboscis form an angle with the line of the abdomen and thorax	Head and proboscis form an angle with the line of the abdomen and thorax
Eggs Individual, boat-shaped, with side floats	Eggs Aggregated into a floating raft	Eggs Individual, surrounded by an air chamber
Larvae No distinct respiratory siphon	Larvae Distinct respiratory siphon	Larvae Short respiratory siphon
Horizontal position in water	Rests at an angle with the surface of the water	Rests almost vertically in the water

Fig. 10. Mosquitoes. Comparison of various stages of anopheline and culicine mosquitoes. 1. <u>Anopheles</u>, A, eggs; B, larva; C, pupa; D, adult; E, wing of adult; F, mouthparts of adult male and female.
2. <u>Aedes</u>, A, eggs; B, larva; C, pupa; D, adult; E, wing of adult; F, mouthparts of adult male and female.
3. <u>Cules</u>, A, typical raft of eggs.

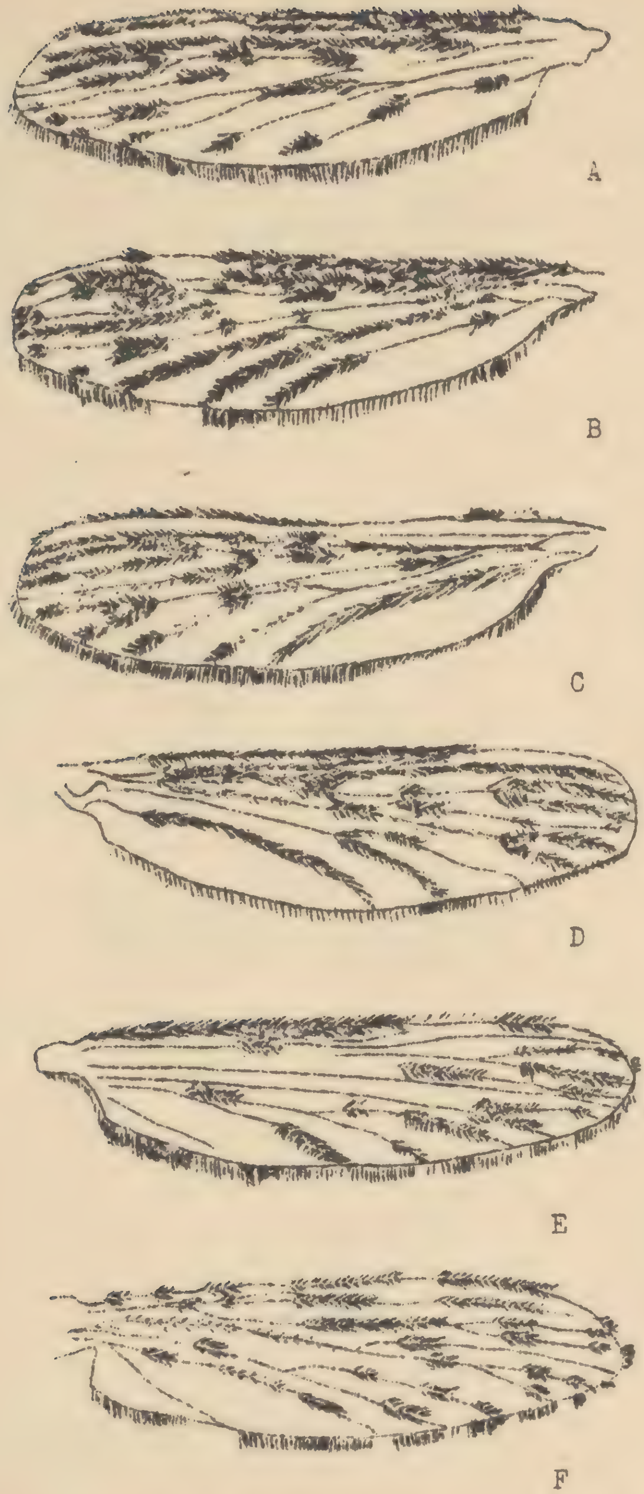
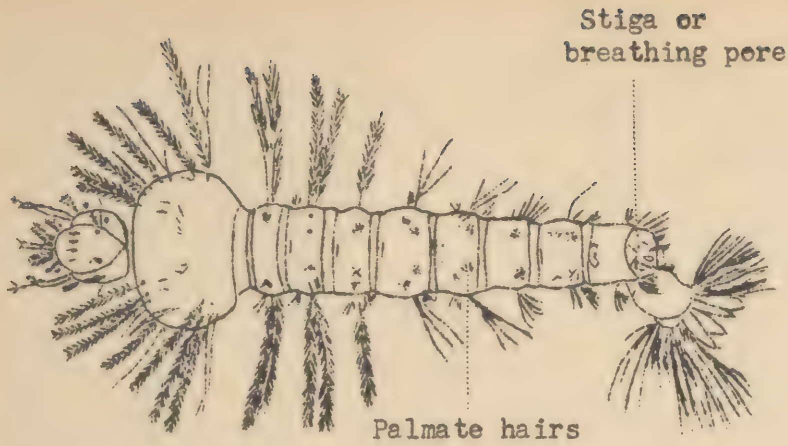
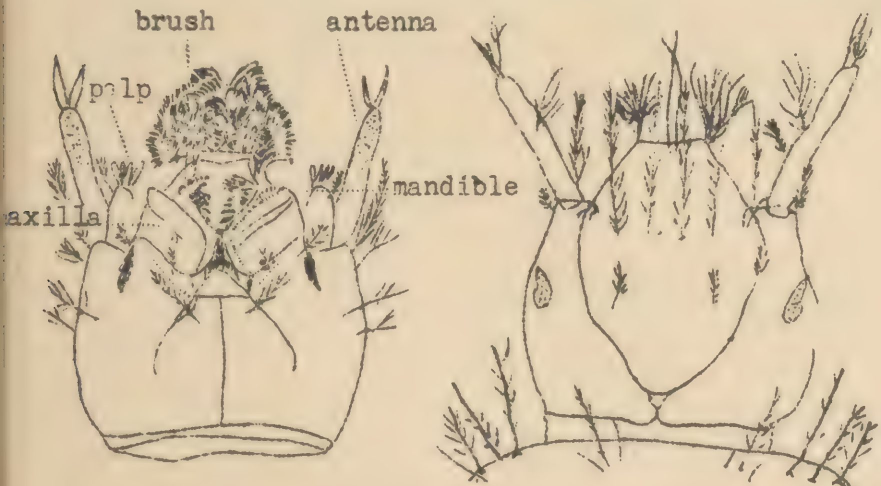


Fig. 11. Wings of *Anopheles* mosquitoes. A, *A.crucians;*
B, *A.punctipennis;* C, *A.maculipennis;*
D, *A. quadrimaculatus;* E, *A. pseudopunctipennis;*
F, *A. albimanus*.

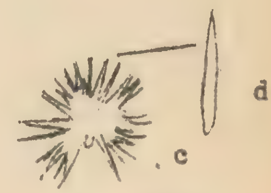


Dorsal view of an Anopheline Larva

A. quadrimaculatus



Ventral and Dorsal views of an Anopheline Larva



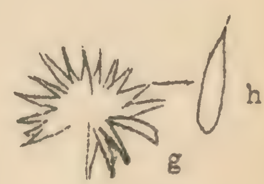
A. albimanus

f

e

A. punctipennis

Hypopygium of male Anopheles albinaus and
A. punctipennis



A. pseudopunclipennis

Fig. 12. Anopheles mosquitoes. Structural characteristics of larva. Hypopygia of males.

Fig. 13. Flies. A, Crane-fly; B, Horse-fly.

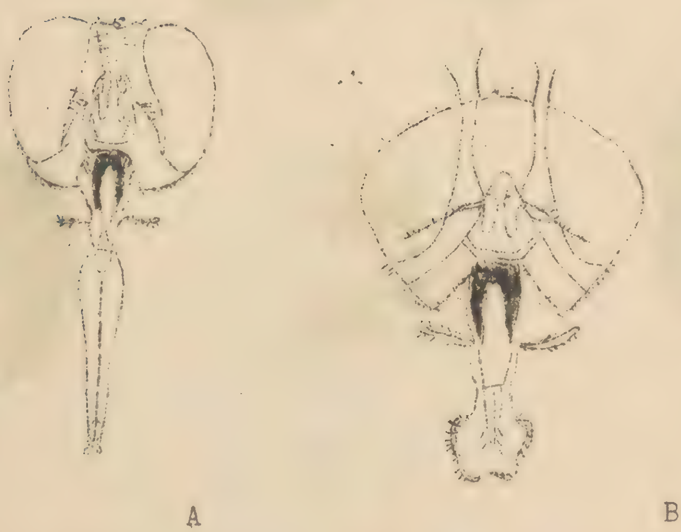


Fig. 14. Fly mouthparts. A, Head of stable-fly showing biting mouthparts; B, Head of house-fly showing non-biting mouthparts.

Fig.15. Stigmal plates of fly larva. A, Blow-fly (Calliphora); B, Green-bottle fly (Lucilia); C, Blue-bottle fly (Cynomyia); D, Screw-worm fly (Cochliomyia); E, Bot fly (Gasterophilus); F, Warble fly (Dermatobia); G, Flesh fly (Sarcophaga); H, Black blow fly (Phormia); I, Biting stable fly (Stomoxys); J, Non-biting Stable fly (Muscina); K, Flesh fly (Wohlfahrtia); L, House fly (Musca); M, Cattle bot fly (Hypoderma); N, Sheep bot fly (Oestris).

Fig. 16. Bedbug (*Heteroptera*).

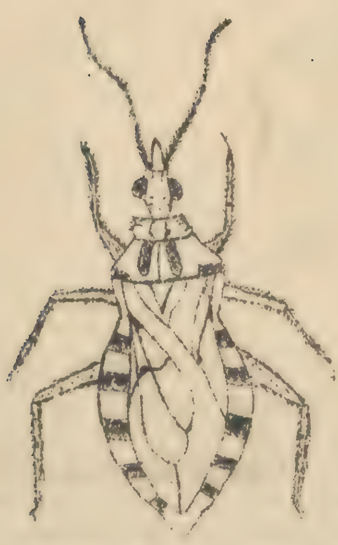


Fig. 17. Kissing-bug (*Heteroptera*).

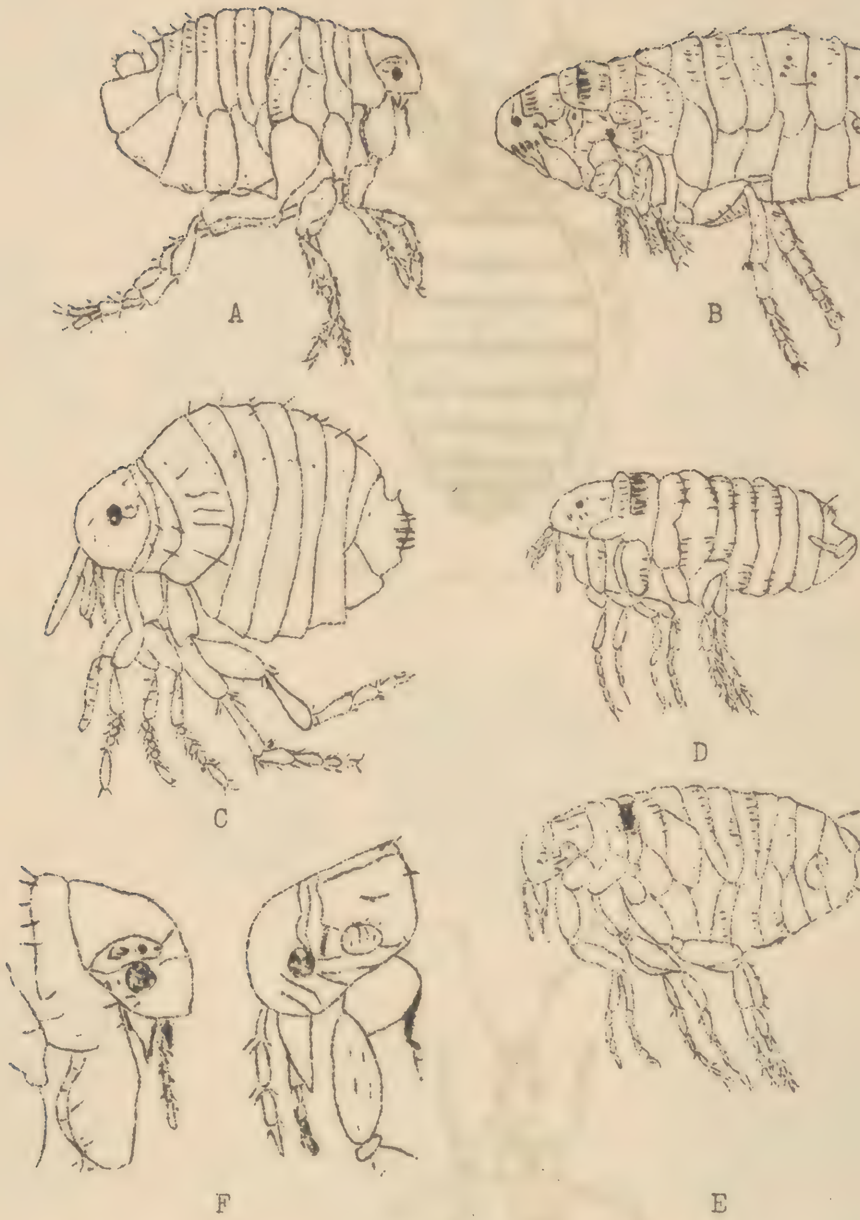


Fig. 18. Fleas (Siphonaptera). A, Human flea (Pulex irritans); B, Dog flea (Ctenocephalus cunis); C, Chicken flea (Echidnophaga gallinacea); D, Temperate zone rat flea (Ceratophyllus fasciatus); E, Tropical rat flea (Xenopsylla cheopis); F, Heads of human flea (left) and tropical rat flea (right) showing arrangement of stout bristles in relation to eyes. (Note that in human flea a stout bristle is directly below the eye, whereas in the tropical rat flea it is in front of the eye.)

INSTRUCTIONS FOR PREPARATION AND SHIPMENT
OF ENTOMOLOGICAL SPECIMENS
AS DIRECTED BY 8TH SERVICE COMMAND LABORATORY
FORT SAM HOUSTON, TEXAS

1. General - One function of the Service Command Medical Laboratory is to accomplish procedures for which a local laboratory is not equipped. One of the phases of laboratory work in which it can be of especial value to the local installations is in the identification of insects and other arthropods which may be of medical importance.

The responsibility of the surgeon of each station and command, with regard to control of mosquitoes (AR 40-205, paragraph 21), includes the investigation of the character of the mosquito population. In this, and also in connection with problems pertaining to other arthropod pests (AR 40-205, paragraphs 22-27 inclusive), the Service Command Laboratory is prepared to assist by making identifications of the organisms involved.

In order that satisfactory reports may be made on samples submitted, co-operation is essential, particularly when specimens must be sent considerable distances. Every effort will be made by this laboratory to make identification of <u>any</u> material sent in, but it is believed that attention to the following instructions will eliminate the bulk of unsatisfactory, unidentifiable specimens.

Improper packing, careless initial handling of specimens, and lack of labeling are the most usual causes for complaint. By observing only a few precautions, these difficulties can be largely eliminated.

2. Preparation and Shipment of Mosquito Specimens.

a. Adult Mosquitoes.

(1) Hand Collections - Hand-caught adult mosquitoes captured by devices such as chloroform tubes, aspirators, (Figures 3 and 4), or insect nets may be killed by exposure to chloroform vapour for five minutes. The period of exposure to strong chloroform vapour should be a full five minutes, inasmuch as those exposed too briefly may be merely stupefied. The insects may then be placed in pill boxes or other suitable containers between layers of cellucotton, crumpled cleansing tissue, or other soft packing materials (Figures 1 and 2). Cotton should never be used, for the loose, fluffy fibers become badly entangled with insect appendages and scales. Too much packing material should not be used - a small amount crumpled into place, and enough to prevent movement of specimens will suffice. Mosquitoes must not be packed in too tightly; each specimen should

be well separated from the next. However, the insects may be packed in layers to increase the capacity of the container. Usually 25 to 50 specimens may be considered as the maximum to be packed in a box. The characters necessary for the proper identification of mosquitoes consists of minute scales, hairs and appendages which are readily broken or scraped off by rough handling; it is therefore desirable to use light forceps in transferring specimens. If no forceps are available, do not use fingers, but slide specimens into the box from the original container used in capture or from a piece of paper on which they have been deposited. Once a batch of specimens has been packed, the box should not be reopened, for the mosquitoes become dry and brittle in a few hours and break with the slightest movement. (At the receiving laboratory, the boxes are placed in a humidifier before opening). The locality of capture, method of taking, name of collector, and date should be written on each box. In addition, an 8th SvC Lab. Entomology form should be filled out and sent in with the sample. Hand-caught mosquitoes are usually taken from diurnal resting places (caves, cattle underpasses, culverts, old buildings, etc.) while in the act of biting, or by insect net in the field. The manner in which the Entomology form is filled will differ slightly for each situation, but is demonstrated by the sample given below.

ENTOMOLOGY

Station Fort Sam Houston, Texas Date 10 June 1943

Collection Data: Map Locality: East P[illegible] ½ Mi. No. of Post

Character of Site:(Woods,Stables,Pond,Marsh,etc.) Cattle Underpass

Date: 10 June 1943 Time: [illegible] A.M. Weather: Overcast

Hand Collection: Type: Diurnal Resting Place - aspirator

If Trap Used: Location of Trap: ______

Time Operated ______ P.M. To ______ A.M. Type of trap: ______

Larval Collection: No. of Dips: ______ No. of Collectors: ______

Remarks: Morning bright & sunny. Overcast by 10:00 AM. Cattle underpass in daily use. Cool to moist.

Collected By: F. Q. Smith, 1st Lt, Sn. C.

(Name, Rank, Designation)

(Report on Reverse Side)

8SC Lab 856-SAASFD-2-29-44-20,000

---

(2) Trap Collections - To impart the best information, traps should be operated regularly throughout the season. Unless unusually great numbers of mosquitoes are entering the traps, the receiving jar (cup) may be emptied once a day - during the early morning. The entire collection should be spread out on large sheets of white paper into a thin layer (use forceps). The mosquitoes may then be recognized and separated from the other insects. The mosquitoes thus set apart should be packed into pill boxes as described in 2a(1) above. Each shipment of mosquitoes should be carefully labeled and accompanied by an 8th SvC Lab. Entomology form. A sample of this form as it should be filled out for trap collections is given below.

ENTOMOLOGY

Station [illegible] Date 8 July 1944

Collection Data: Map Locality: [illegible]
Character of Site: (Woods, Stables, Pond, Marsh, etc.) [illegible]
Date: [illegible] Time: ---- Weather: [illegible]
Hand Collection; Type: ____
If Trap Used; Location of Trap: [illegible]
Time Operated 8 P.M. To 8 A.M. Type of Trap: New Jersey
Larval Collection: No. of Dips: ____ No. of Collectors: ____
Remarks: [illegible]

Collected By: [illegible]
(Name, Rank, Designation)

(Report on Reverse Side)

8SC Lab 856-SAASFD-2-29-44-20,000

(3) Living Adults - When in special instances it may be desirable to send in a few matured females to be examined for natural infection with malarial parasites, they may be shipped short distances by using the bottle shown in Fiture 6, if the glass tube is covered at its lower end with gauze or gauze is substituted for the cork. When such special examinations are requested, complete data concerning the point of collection, attendant circumstances and the reason for making the request should accompany the shipment. A few blades of grass or strips of crumpled paper are included in the container to serve as supports or perches for the insects. In the transport of adults there is no need of including the water in the shipping container as shown in Figure 6.

b. Larval Mosquitoes.

(1) Larvae Sent in After Killing - Larvae are chiefly identified by the characteristics of hairs on body and head, and other similar minute differences. Hence, these, as well as the adult specimens, must be handled very carefully. Since characters of young larvae are not adaptable to ready identification, large larvae should always be included, e.g., the stage immediately preceding the pupa and emergence of the adult. If possible the larvae should be killed by dropping into hot water 150°F. (not boiling water) or into 70% alcohol. Following this treatment they may be forwarded in small shell vials containing 70 - 95% alcohol or 10% formalin (a 1 to 10 dilution of the common commercial formalin). The vial should be almost completely filled with liquid, allowing a moderate sized bubble for expansion; this prevents undue splashing and violent agitation of specimens, or better yet, the vial may be prepared as shown in Figure 5. Each vial should contain within (not glued to the outside) an adequate label identifying the catch. A pencil should be used to fill in the information to insure permanency. Larvae from one source only should be included in a single container. The form accompanying the shipment and identified by the same number as that within the vial should be filled out as shown in the sample below.

ENTOMOLOGY

Station [illegible] Date 8 July 1944

Collection Data: Map Locality: 1/2 mi. South of Vet. Hosp

Character of Site: (Woods, Stables, Pond, Marsh, etc.) [illegible] Creek

Date: 8 July 1944 Time: 10:30 A.M. Weather: Sunny

Hand Collection: Type: ______

If Trap Used; Location of Trap: ______

Time Operated _____ P.M. To ____ A.M. Type of Trap: ______

Larval Collection: No. of Dip: 100 No. of Collectors: 2

Remarks: [illegible] 30 minutes [illegible] mats throughout [illegible]

Collected By: [illegible]

(Name, Rank, Designation)

(Report on Reverse Side)

8SC Lab 856-SAASFD-2-29-44-20,000

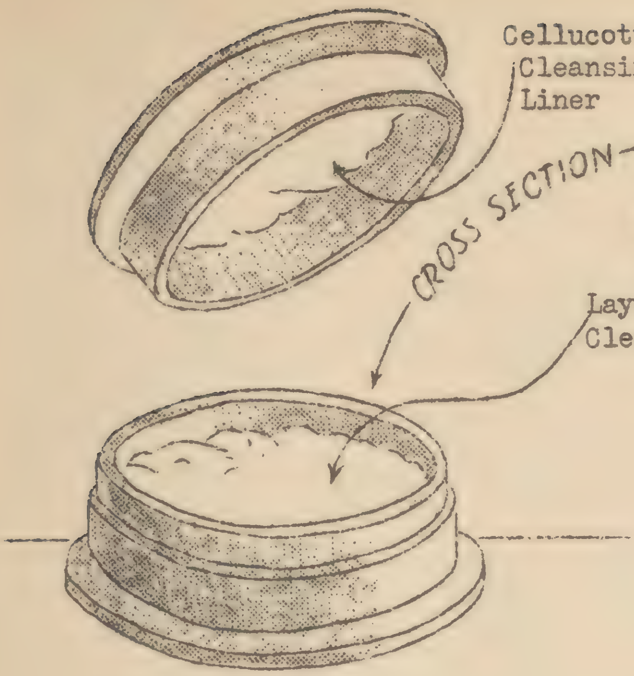
Caution: Postal authorities require that vials of this kind, containing liquids, be inclosed in a screw-capped tin cylinder, which in turn is placed inside a paper mailing carton.

(2) Live Larvae - It is often desirable to forward live specimens of the larvae for identification. Live specimens of this kind may be reared to the adult stage in the laboratory and identification is facilitated. Large larvae are preferable, as less time will be required for emergence and mortality is reduced. A 120 milliliter wide mouth histological bottle, which has been modified as shown in Figure 6 by inserting a glass tube in the cork, is used as a shipping container. Water from the natural habitat is used to ship the larvae and the container is filled to approximately 1/4 its capacity. Not more than 25 larvae should be placed in each container. Every effort should be made to insure promptness in transit and that the container is kept cool. At the height of the summer season it is probably inadvisable to ship live larvae more than 200 miles. The bottle is shipped in the mailing case described in the footnote of 2b(1) above. Proper labeling and identification of the container is essential and larvae from a single source only should be included in each container. The information to be included with the shipment should be the same as that listed in Section 2b(1).

3. Preparation and Shipment of Arthropods Other Than Mosquitoes.

a. Flies and gnats, such as horse flies, deer flies, black flies (Buffalo gnats), biting muscids, etc., may be killed and packed for shipment in the manner described for mosquitoes in Section 2a(1) above.

b. Ticks, mites, bedbugs, lice, fleas and Triatoma bugs may be killed directly upon collection by plunging into 70% alcohol, and preserved for shipment in the same fluid. Before placing in shipping container, a plug of absorbent cotton should be forced down the vial until it almost contacts the specimens. This is done to prevent movement among specimens which may damage structures needed for identification. The vial is then filled with 70% alcohol to a point 1/4 inch below the stopper, and a label is inserted. This label should be made out in pencil and should state the locality, host (if any), collector, and date of collection. The vial should be mailed in a standard mailing tube for liquids as indicated in footnote caution, Section 2b(1).



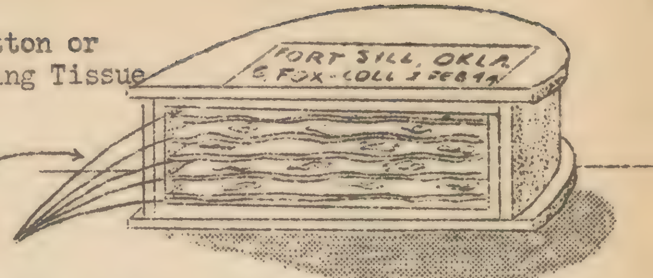
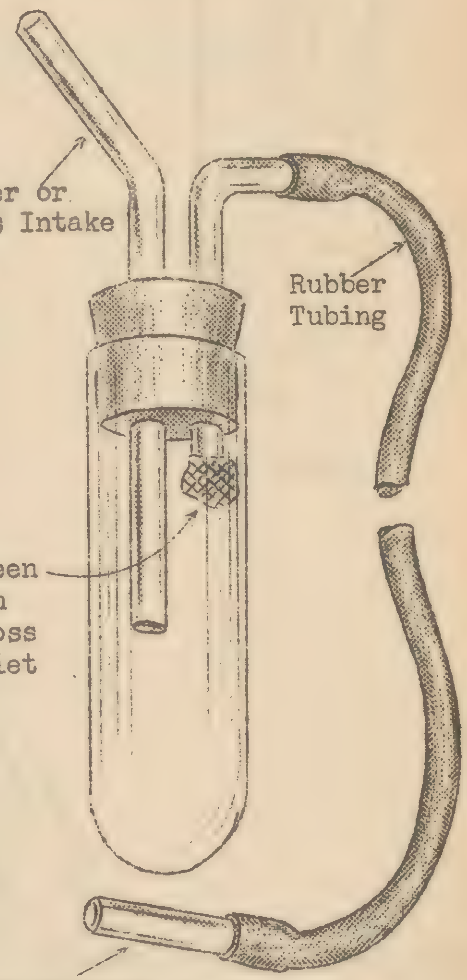


Figure 2.

Figure 1.



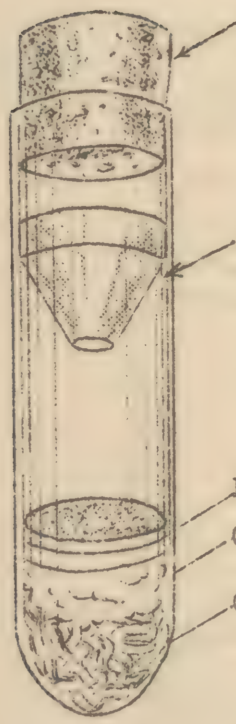


Figure 3.

Figure 4.

ASF, Eighth Service Command Laboratory
Fort Sam Houston, Texas
March, 1944

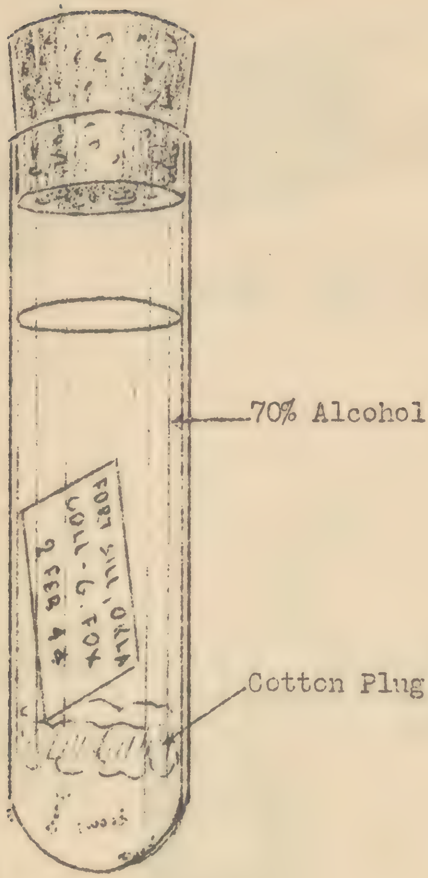


Figure 5.

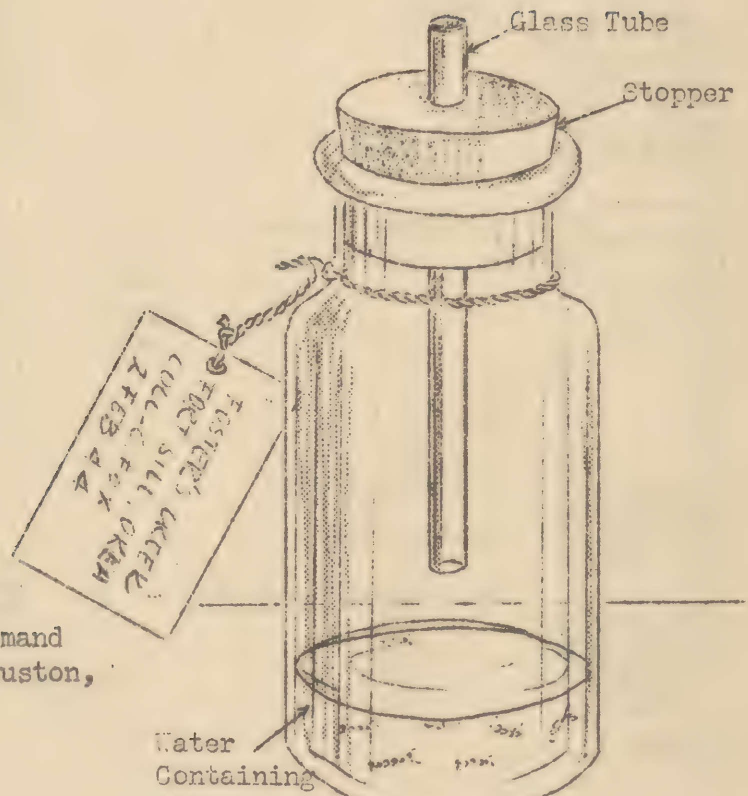


Figure 6.

ASF, Eighth Service Command
Laboratory, Fort Sam Houston,
Texas, March, 1944

REFERENCES

War Department Training Manual 8-227

Medical Protozoology and Helminthology - Army Medical School

Laboratory Methods of the United States Army - Simmons

Clinical Parasitology, 3d Edition - Craig and Faust

Clinical Diagnosis by Laboratory Methods, 8th Edition - Todd & Sanford

Practical Bacteriology, Hematology and Parasitology, 9th Edition - Stitt, Clough & Clough

Approved Laboratory Technic, 3d Edition - Kolmer & Boerner

Textbook of Clinical Pathology - Kracke

Synopsis of Clinical Laboratory Methods - Bray

All illustrations supplied by courtesy of Army Medical Museum, Washington, D. C.